REMARKS

The Specification has been amended to include sequence identification numbers which were omitted at the time of filing.

Claims 1, 5-7, and 19-23 were pending. Claims 1 and 5 are amended herein. Support for the amendment of claim 5 is found throughout the specification at, *e.g.*, page 10, line 16 to page 11, line 2. Claim 20 is canceled herein. Claims 1, 5-7, 19, and 21-23 are currently pending. No claim is allowed.

Formal Matters

Applicants gratefully acknowledge the withdrawal of finality of the Action dated March 6, 2003 and the entry of the amendment received May 6, 2003.

Applicants acknowledge the acceptance of the Terminal Disclaimer received November 27, 2002.

The Examiner requests the new abstract be presented on a separate page. Applicants note that the amended Abstract was presented on a separate page in the Amendment filed May 6, 2003. Nonetheless, Applicants have provided a clean copy of the Abstract (as amended) on a separate page herein.

The Examiner requests that the status of related application be updated in the specification. The status of the related applications was updated in the Amendment filed on May 6, 2003.

Applicants gratefully acknowledge the consideration of the Information Disclosure Statements received on March 14, 2000 and September 10, 2001 and the withdrawal of the written description and enablement rejections under 35 U.S.C. § 112, first paragraph.

The Office requested information regarding as to how the present application differs from Serial No. 09/361,775, now U.S. Patent 6,410,512, and 09/113,947, now U.S. Patent No. 6,462,019 to confirm the priority date. Applicants believe that the Examiner has the resources to fulfill his burden to determine priority. Nonetheless, Applicants provide a brief summary of each of these patents, both of which were prosecuted and issued by Examiner Gitomer. U.S. Patent No. 6,410,512 relates to methods of methods of stimulating hair growth using the proteasomal inhibitor, PS1. U.S. Patent No. 6,462,019 relates to methods of stimulating bone growth using the proteasomal inhibitor, PS1. The instant application discloses additional data regarding the stimulation of bone growth with various proteasomal inhibitors relative to U.S. Patent Nos. 6,462,019 and 6,410,512.

The Office requested the identity of any related applications, abandoned, pending or allowed. The co-pending, related Application Serial No. 09/558,973 (a continuation-in-part of the instant application) (now allowed), relates to methods of stimulating bone growth using various proteasomal inhibitors including PS-431, NLVS, and warhead compounds. The co-pending, related Application Serial No. 09/695,807 (a continuation-in-part of the instant application) relates to methods of stimulating bone growth using compounds that are proteasomal inhibitors. Applications Serial No. 10/050,425, 10/050,633 (now allowed), and 10/052,832 also are related to the instant application and have claims directed to the stimulation of hair growth using various inhibitors of proteasomal activity.

Applicants gratefully acknowledge the acceptance of the request for correction of the inventor's name. Applicants are unaware of any requirement that a single copy of the declaration be signed by each inventor. Therefore, Applicants believe the submitted declarations have properly provided the necessary signatures. Nonetheless, in an effort to expedite prosecution, Applicants submit a substitute declaration herein as Exhibit A.

Applicants appreciate the identification of the subject matter of claims 20 and 21 as allowable if rewritten in independent form.

sd-168865

The Examiner states that the about 85 references submitted would appear to be directed to non-analogous art, noting that none appear to be directed to hair growth treated by any of the classes of claimed compounds. Applicants respectfully note the claimed methods of this application are not drawn to methods of stimulating hair growth, but to methods of stimulating **bone growth**. Thus, it does not appear that the comments made by the Examiner are addressed to the instant application.

The Examiner states that the application fails to comply with the requirements of 37 C.F.R. §§ 1.821 - 1.825 for one or more reasons set forth in the attached "Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosure." Applicants note that no such Notice to Comply was included with the response. Nonetheless, the Specification is amended herein to include sequence identification numbers. Applicants have also included a Statement Pursuant to 37 C.F.R. § 1.823(b), a paper copy and a labeled, computer readable copy of the Sequence Listing included in the instant application.

Claim 1 is amended and Claim 20 is canceled herein as the compound, PTX, is not a proteasomal inhibitor. Subsequent work by Applicants and independent observations by others indicate that PTX is not a proteasomal inhibitor, and therefore this compound is deleted from claim 1 and claim 20 is canceled. An unexecuted declaration by Dr. Gregory Mundy attesting to the absence of proteasomal activity by PTX is attached as Exhibit B. An executed declaration will follow under separate cover.

Objection to the Specification

The Actions objects to the specification as failing to provide proper antecedent basis for the claimed subject matter, citing 37 C.F.R. § 1.75(d)(1) and MPEP § 608.01(o), because the specification allegedly fails to provide written description for the presently claimed peptidyl aldehyde. Applicants traverse this objection.

Applicants have amended the specification herein at page 28 to include the language of the original claim 4, which reads as follows:

4. The method of claim 1 wherein said compound is lactacystin, a peptidyl aldehyde, PTX, or epoxomicin.

Applicants believe that this amendment provides the necessary antecedent basis for the term "peptidyl aldehyde."

In light of the above remarks, Applicants respectfully submit that the objection to the specification have been overcome. Therefore, Applications request the withdrawal of the objection.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claim 5 is rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite. According to the Examiner, the term "post-plastic surgery" is indefinite regarding what kind of condition may be intended. Applicants traverse this rejection.

Applicants submit that the term "post-plastic surgery" is sufficiently definite in light of the disclosure in the specification. *See* specification at page 10, line 16 to page 11, line 2. Nonetheless, claim 5 is amended herein to further clarify the term "post-plastic surgery" as relating to conditions where the promotion of bone healing following plastic surgery is desirable, and therefore this rejection is moot.

In view of the above, Applicants request the withdrawal of this rejection.

Rejection Under 35 U.S.C. § 102 (b)

Claims 1, 5-7, 19, and 22-23 are rejected under 35 U.S.C. § 102 (b) as allegedly being anticipated by Tanihara (JP 6025288). According to the Examiner, Tanihara teaches peptidyl aldehydes for treating osteoporosis as well as the simultaneous administration of fluoride as an anti-resorptive agent. Applicants traverse this rejection.

Applicants respectfully submit that Tanihara fails to teach each and every element of the claimed invention and therefore does not anticipate the claimed methods. More specifically, Tanihara fails to teach the use of compounds that are proteasomal inhibitors to stimulate bone growth, and subsequently treat conditions such as osteoporosis. In fact, Tanihara is completely silent with regards to peptides, particularly peptidyl aldehydes, that are inhibitors of proteasomal activity. Tanihara discloses peptides with transforming growth factor (TGF)-β activity to treat various indications including osteoporosis. *See, e.g.*, Tanihara, at ¶1. Tanihara discloses the

making and using of peptides with TGF-β activity that are less immunogenic than the native protein. It is well known in the art that TGF-β mediates its activities through the binding and activation of a cellular receptor, the TGF-β receptor, to initiate an intracellular signaling cascade. *See, e.g.*, Howe, P.H. *Transforming Growth Factor* β IN THE CYTOKINE HANDBOOK 1119, 1121 (4th Ed. 2003) (stating that the "cellular actions of TGF-β are mediated through binding to ... cell surface receptors"). Therefore, Tanihara discloses synthetic peptides that emulate TGF-β in biological activity, presumably through binding to the TGF-β receptor. Applicants are unaware of any scientific evidence that TGF-β functions as a proteasomal inhibitor, and neither the Examiner nor Tanihara provide such evidence. Moreover, the art teaches that TGF-β is anti-proliferative for hair follicles. *See e.g.*, Blessing et al., *Genes & Develop.* 7:204-15 (1993) (cited in Information Disclosure Statement submitted on March 10, 2000 in instant application.). Furthermore, contrary to the express assertion of the Examiner, this is no disclosure whatsoever regarding the simultaneous administration of fluoride as an anti-resorptive agent. The only mention of fluoride is in a description of the solid phase synthesis of the peptide with TGF-β activity while there is no mention of anti-resorptive agents at all. In view of the complete absence of any disclosure regarding the use of peptidyl aldehydes that are proteasomal inhibitors to stimulate bone growth, Tanihara fails to anticipate the claimed methods.

Therefore, Applicants respectfully request the withdrawal of the rejection.

CONCLUSION

Applicants submit that the objections and the rejections under 35 U.S.C. §§ 102 (b) and 112, second paragraph have been overcome by the above remarks. Early allowance of the remaining pending claims 1, 5-7, 19, and 21-23 is respectfully requested. In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 43272002621. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: March 8, 2004

Respectfully submitted,

By _____

Laurie L. Hill, Ph.D.
Registration No.: 51,804
MORRISON & FOERSTER LLP
3811 Valley Centre Drive, Suite 500
San Diego, California 92130
(858) 720-7955

sd-168865

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

SUBSTITUTE DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE AND HAIR GROWTH

the specification of which was filed on October 20, 1999 as Application No. 09/421,545 and amended on October 18, 2001, November 19, 2002, and May 6, 2003.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein.

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

FOREIGN PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

[x] no foreign applications have been filed

[ ] foreign application have been filed as follows:

EXHIBIT A

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing	Priority Claimed Under 35 USC 119
			___ Yes No ___
			___ Yes No ___
			___ Yes No ___

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing

CLAIM FOR BENEFIT OF EARLIER U.S. PROVISIONAL APPLICATIONS

I hereby claim priority benefits under Title 35, United States Code §119(e), of any United States provisional patent application(s) listed below:

[x] no U.S. provisional applications have been filed.

[ ] U.S. provisional application have been filed as follows:

Application Number	Date of Filing	Priority Claimed Under 35 USC 119
		___ Yes No ___
		___ Yes No ___
		___ Yes No ___

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information that is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56 which became available to me between the filing date of the prior application and the national or PCT international filing date of this application:

☐ no U.S./PCT applications have been filed.

☒ U.S./PCT application have been filed as follows:

Application Number	Date of Filing	Status (Patented/Pending/Abandoned)
09/361,775	July 27, 1999	Patented
09/113,947	July 10, 1998	Patented

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint:

All practitioners at Customer Number 25225

all of **Morrison & Foerster LLP**, 3811 Valley Centre Drive, Suite 500, San Diego, California 92130, jointly, and each of them severally, my attorneys at law/patent agent(s), with full power of substitution, delegation and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, and to transact all business in the U. S. Patent and Trademark Office connected therewith.

Please mail all correspondence to Peng Chen, whose address is:

Morrison & Foerster LLP
3811 Valley Centre Drive, Suite 500
San Diego, California 92130

Please direct telephone calls to: Peng Chen at (858) 720-5117.

Please direct facsimiles to: (858) 720-5125

Full name of sole or first inventor Gregory R. MUNDY	
Sole or first inventor's signature	Date 10/25/03
Residence San Antonio, Texas	
Citizenship US	
Mailing Address 3719 Morgan's Creek San Antonio, Texas 78230	

Full name of second inventor, if any I. R. GARRETT	
Second inventor's signature	Date 10-23-2003
Residence San Antonio, Texas	
Citizenship Australia	
Mailing Address 16729 Windjammer Court San Antonio, Texas 78023	

Full name of third inventor, if any Jorge G. ROSSINI	
Third inventor's signature	Date 10-23-2003
Residence San Antonio, Texas	
Citizenship Chile	
Mailing Address 7207 Snowden Rd., #505B San Antonio, Texas 78240	

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail, Airbill No. EV 272143888 US in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

3-8-2004
Date

Rhea Amid

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Gregory R. Mundy *et al.*

Serial No.: 09/421,545

Filing Date: October 20, 1999

For: INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE GROWTH (AS AMENDED)

Examiner: R.J. Gitomer

Group Art Unit: 1651

DECLARATION OF GREGORY R. MUNDY PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Gregory R. Mundy, declare as follows:

1. I am one of the co-inventors of the subject matter claimed in the above-referenced application.

2. Other co-inventors and I have conducted experiments demonstrating that pentoxifylline (PTX) is not a proteasomal inhibitor. The experimental results demonstrating the lack of proteasomal activity by PTX are set forth in the following paragraphs and in the attached Figure 1.

EXHIBIT B

3. Proteasomal activity was determined using the assays described in the instant specification. *See* specification, at page 17, lines 8-20 and Example 5. Briefly, the effects of inhibitors of proteasomal activity were assessed using a fluorogenic peptide chymotryptic substrate. The 20S proteasomes from the methanoarchaeon *Methanosarcina thermophila* produced in *E. coli* and the substrate Suc-Leu-Leu-Val-Tyr-AMC were obtained from Calbiochem-Novabiochem Corp. Briefly, serial dilutions of the inhibitor to be tested were mixed with proteasome solution at a proteasome concentration of 0.01 mg/ml. After 30 minutes of incubation at 37°C, substrate solution at a final concentration of 20-30 μg/ml was added; the mixture was incubated at 37°C and then read at 15, 30, and 60 minutes in a Titertek Fluoroskan II (MTX Lab Systems, Inc., Vienna, Virginia, USA). Five different compounds were tested using increasing concentrations of each compound.

4. Figure 1 shows the results the inhibitory activity of several compounds in the proteasomal activity assay performed as described in paragraph 3. MG262, PS1, and MG132 inhibited proteasomal activity as evidenced by the decreasing amount of fluorescence detected as the concentration of the test compound increased. Maximal inhibition occured for MG262 at 1μM, for PS1 at 10 μM, and for MG132 at about 50 μM. However, the NF-κB inhibitors, α-benzoylamino-1,4-naphthoquinone (PPM18) and pentoxyfilline (PTX), failed to inhibit proteasomal activity. Thus, our data demonstrates that PTX fails to inhibit proteasomal activity.

5. To my knowledge, there has been only one report identifying PTX as a proteasomal inhibitor. The report identifying PTX as a proteasomal inhibitor is Combaret et al., *Mol. Biol. Rep.* 26:95-101 (1999). This reference is cited in the original disclosure as filed at page 29, lines 17-20 as it was published prior to the submission of the application. However, based on our testing of PTX, I do not believe that Combaret report is accurate.

6. The other publications I have reviewed regarding the functional activity of PTX do not identify PTX as a proteasomal inhibitor. PTX is alternatively classified as a NF-κB inhibitor or a phosphodiesterase inhibitor. For example, Chen et al. discussed the action of PTX on TNF-a mediated activity in vascular smooth muscle cells. *See* Exhibit C. In their discussion

on pages 954-956, the various activities of PTX were disclosed only as NF-κB inhibition and phosphodiesterase inhibition. Likewise in Lee et al. disclosed PTX as having only two identified inhibitory functions - the inhibition of NF-κB activity and of phosphodiesterase activity. *See* Exhibit D at, *e.g.*, Abstract.

7. In view of our own data demonstrating that PTX fails to inhibit proteasomal activity and the inability of other skilled artisans to reproduce the observations reported by Combaret, I do not believe that PTX is a proteasomal inhibitor.

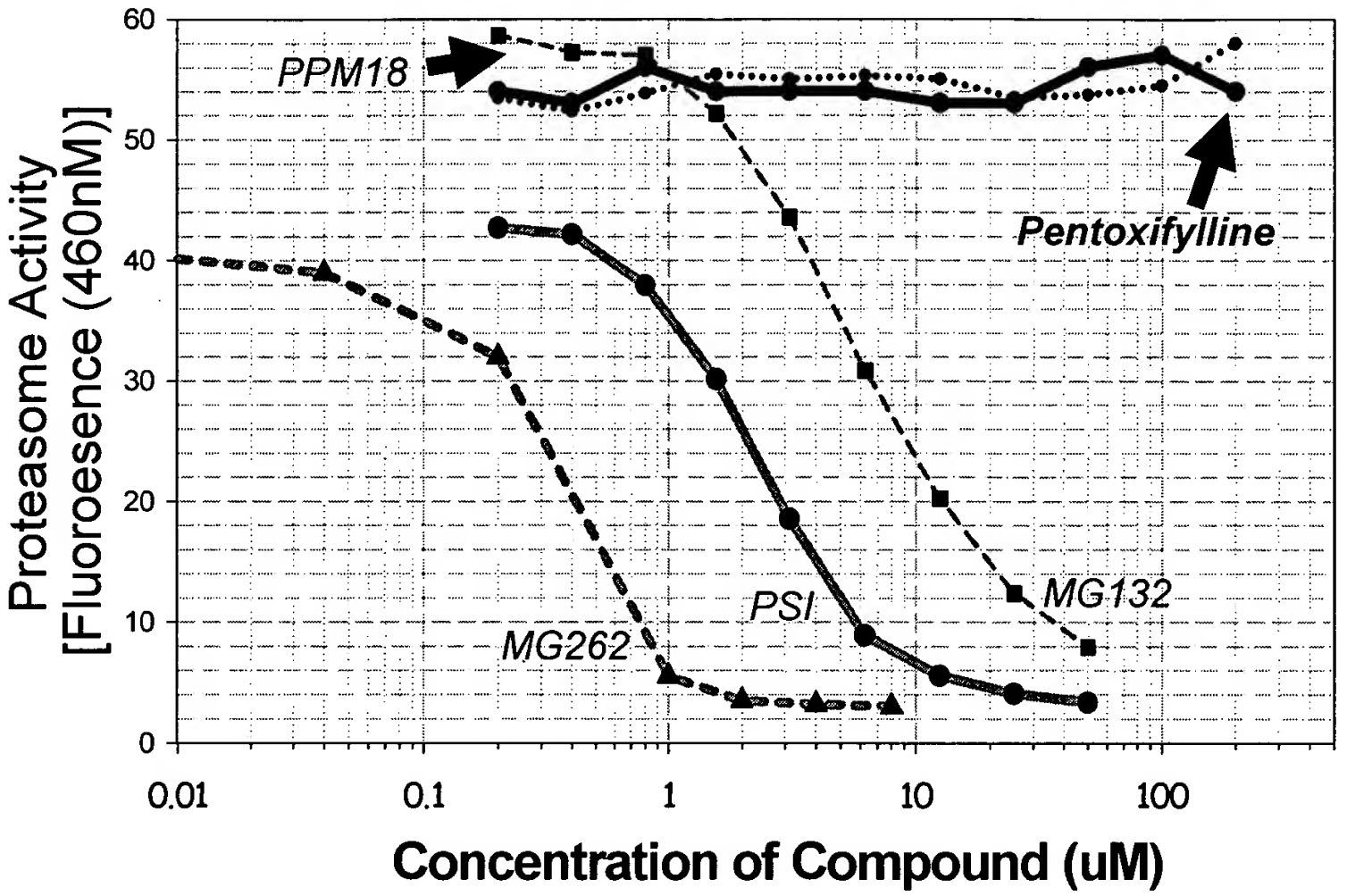
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at San Antonio, Texas, on _____, 2004.

_____

Gregory R. Mundy

FIGURE 1.



British Journal of Pharmacology (2003) 138, 950–958

www.nature.com/bjp

Inhibition by pentoxifylline of TNF-α-stimulated fractalkine production in vascular smooth muscle cells: evidence for mediation by NF-κB down-regulation

[1]Yung-Ming Chen, [1]Chao-Jung Tu, [1]Kung-Yu Hung, [1]Kwan-Dun Wu, [1]Tun-Jun Tsai & *,[1]Bor-Shen Hsieh

[1]Department of Internal Medicine, National Taiwan University Hospital and College of Medicine National Taiwan University, Taipei, Taiwan

1 Fractalkine is a CX_3C chemokine for mononuclear leukocytes that is expressed mainly by vascular cells, and regulated by pro-inflammatory cytokines. This study investigated signal transduction mechanisms by which tumor necrosis factor (TNF)-α stimulated fractalkine expression in cultured rat vascular smooth muscle cells (VSMCs), and the modulatory effect of a haemorrheologic agent, pentoxifylline, on its production.

2 TNF-α (1–50 ng ml^{-1}) stimulated fractalkine mRNA and protein expression in concentration- and time-dependent manners. Pretreatment with calphostin C (0.4 μM, a selective inhibitor of protein kinase C (PKC), and PD98059 (40 μM), a specific inhibitor of p42/44 mitogen-activated protein kinase (MAPK) kinase, attenuated TNF-α-stimulated fractalkine mRNA and protein expression. In contrast, H-89 (2 μM), a selective inhibitor of cAMP-dependent protein kinase, wortmannin (0.5 μM), a selective inhibitor of phosphatidylinositol 3-kinase, and SB203580 (40 μM), a specific inhibitor of p38 MAPK, had no discernible effect.

3 The ubiquitin/proteosome inhibitors, MG132 (10 μM) and pyrrolidine dithiocarbamate (200 μM), suppressed activation of NF-κB as well as stimulation of fractalkine mRNA and protein expression by TNF-α.

4 TNF-α-activated phosphorylation of PKC was blocked by calphostin C, whereas TNF-α-augmented phospho-p42/44 MAPK and phospho-c-Jun levels were reduced by PD98059. Neither calphostin C nor PD98059 affected TNF-α-induced degradation of I-κBα or p65 nuclear translocation.

5 Pretreatment with pentoxifylline (0.1–1 mg ml^{-1}) decreased TNF-α-stimulated fractalkine mRNA and protein expression, which was preceded by a reduction in TNF-α-activated phosphorylation of PKC, p42/44 MAPK and c-Jun as well as degradation of I-κBα and p65/NF-κB nuclear translocation.

6 These data indicate that activation of PKC, p42/44 MAPK kinase, and NF-κB are involved in TNF-α-stimulated fractalkine production in VSMCs. Down-regulation of the PKC, p42/44 MAPK, and p65/NF-κB signals by PTX may be therapeutically relevant and provide an explanation for the anti-fractalkine effect of this drug.

British Journal of Pharmacology (2003) **138,** 950–958. doi:10.1038/sj.bjp.0705088

Keywords: Atherosclerosis; fractalkine; mitogen-activated protein kinase; protein kinase C; transcription factor(s); vascular smooth müscle cells

Abbreviations: AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's media; FCS, foetal calf serum; H-89, N-[2-bromocinnamyl (amino)ethyl]-5-isoquinolinesulphonamide; I-κBα, inhibitory protein of NF-κB; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PI 3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTX, pentoxifylline; RT–PCR, reverse transcription-polymerase chain reaction; TNF-α, tumour necrosis factor-α; VSMCs, vascular smooth muscle cells

Introduction

Inflammatory phenomena at sites of atherosclerotic plaques are increasingly thought to be major determinants of the progression and clinical outcome of atherosclerotic diseases (Ross, 1999). Inflammation involved in atherogenesis is mediated largely by monocyte-derived macrophages and specific subtypes of T cells, which emigrate from the blood and multiply within the atherosclerotic lesions (Gerszten *et al.*, 2000). Attraction of mononuclear leukocytes to atherosclerotic lesions involves a series of complex interactions between cellular adhesion molecules and chemotactic cytokines expressed by leukocytes and vessel cells (Braun *et al.*, 1999; Price & Loscalzo, 1999; Reape & Groot, 1999; Sasayama *et al.*, 2000). Recently, a novel CX_3C chemokine known to capture and direct migration of mononuclear leukocytes has been identified. This chemokine is known as fractalkine (Bazan *et al.*, 1997), and it acts primarily as an

*Author for correspondence at: Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, 10016, Taiwan; E-mail: bshsieh@ha.mc.ntu.edu.tw

EXHIBIT C

adhesive molecule in capturing circulating monocytes, T cells, and natural killer cells that express its cognate receptor, CX_3CR1 (Imai *et al.*, 1997; Fong *et al.*, 1998). When cleaved proximal to the membrane by metalloproteinases, cell-bound fractalkine is shed and functions as a soluble chemoattractant (Chapman *et al.*, 2000a; Garton *et al.*, 2001). In humans, high levels of fractalkine mRNA have been localized to advanced atherosclerotic lesions (Greaves *et al.*, 2001). Moreover, CX_3CR1 V249I polymorphism is associated with a significant decrease in fractalkine-binding affinity as well as a reduced risk of coronary artery disease and improved endothelium-dependent vasodilation (Moatti *et al.*, 2001; McDermott *et al.*, 2001). Together, these data suggest that the fractalkine-CX_3CR1 system is involved in the pathogenesis of atherosclerotic disease (Umehara *et al.*, 2001a).

Fractalkine was originally discovered in human umbilical vein endothelial cells upon stimulation with tumour necrosis factor (TNF)-α or interleukin-1β (Bazan *et al.*, 1997). In addition, fractalkine production has been found in non-endothelial cells which include neurons and astrocytes in the central nervous system (Harrison *et al.*, 1998; Schwaeble *et al.*, 2001), dendritic cells within the tonsil and skin (Kanazawa *et al.*, 1999; Papadopoulos *et al.*, 1999), and epithelial cells in the gut (Lucas *et al.*, 2001). More recently, fractalkine expression has been induced in TNF-α-activated vascular smooth muscle cells (VSMCs) (Ludwig *et al.*, 2002). The precise signal pathways leading to fractalkine induction have not been fully elucidated. In cultured endothelial cells, Garcia *et al.* (2000) suggest that fractalkine production by TNF-α is nuclear factor (NF)-κB-dependent. However, they did not provide additional information regarding other TNF-α-activated signalling pathways that may mediate fractalkine production. TNF-α is a pleiotropic cytokine that can initiate distinct cellular signals upon binding to its receptors. In addition to the well-known stimulatory effect on NF-κB activity (Malinin *et al.*, 1997), TNF-α also activates an array of signalling pathways, notably the cAMP-dependent protein kinase (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-K), and mitogen-activated protein kinases (MAPKs) (Vilcek & Lee, 1991; Heller & Kronke, 1994). Stimulation of these protein kinases leads to activation of distinct transcription factors that includes c-Jun/activator protein (AP)-1 and NF-κB (Berghe *et al.*, 1998; Lallena *et al.*, 1999; Reddy *et al.*, 2000). The present study investigated the signal transduction mechanisms that mediated TNF-α-stimulated fractalkine production in VSMCs, and the modulation of fractalkine production by pentoxifylline (PTX), a clinically available haemorrheologic agent which we have demonstrated exerts anti-proliferative and anti-fibrogenic effects on VSMCs (Chen *et al.*, 1999).

In this study, we show that TNF-α activation of PKC, p42/44 MAPK, and NF-κB are involved in TNF-α-stimulated fractalkine expression in VSMCs. Down-regulation of the PKC, p42/44 MAPK, and NF-κB signals may contribute to PTX inhibition of fractalkine gene transcription by TNF-α.

Methods

Reagents

Dulbecco's modified Eagle's media (DMEM), foetal calf serum (FCS), and other tissue culture reagents were obtained from Gibco BRL (Rockville, MD, U.S.A.). Culture flasks and plates were purchased from Costa Corning (Cambridge, MA, U.S.A.). PTX was purchased from Sigma (St. Louis, MO, U.S.A.). N-[2-bromocinnamyl (amino)ethyl]-5-isoquinolinesulphonamide (H-89), calphostin C, PD98059, SB203580, wortmannin, MG132, pyrrolidine dithiocarbamate (PDTC), GM 6001, and actinomycin D were obtained from Calbiochem (La Jolla, CA, U.S.A.). Recombinant rat TNF-α and goat anti-rat fractalkine were obtained from R & D Systems (Minneapolis, MN, U.S.A.). Rabbit anti-p42/44 MAPK, and mouse anti-phospho-p42/44 MAPK, anti-phospho-PKC(pan) (including α, β_I, β_{II}, ε, η and δ isoforms) were obtained from New England BioLab (Beverly, MA, U.S.A.). Mouse anti-phospho-c-Jun, and rabbit anti-c-Jun, anti-PKC β_{II}, anti-PKCζ/ι, anti-phospho-PKCζ/ι, anti-p65/NF-κB and anti-inhibitory protein of NF-κB (I-κB)α were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Mouse anti-β-actin was obtained from Sigma. All chemicals used for total RNA isolation, reverse transcription-polymerase chain reaction (RT–PCR), Northern blot analysis and Western blot analysis were of molecular grade and were obtained from Sigma or Roche Molecular Biochemicals (Mannheim, Germany) unless otherwise specified.

Cell culture

Primary culturing of rat aortic VSMCs was performed as described previously (Chen *et al.*, 1999). Cells were characterized as VSMCs on the basis of the presence of α-smooth muscle actin staining with the avidin-biotin-peroxidase method, using diaminobenzidine as the chromogen. VSMCs between 10–20 passages were used and grown in DMEM containing 10% FCS.

TNF-α-stimulated fractalkine expression by VSMCs: Regulation through protein kinases and NF-κB, and modulation by PTX

These experiments were performed to determine the regulatory role of various protein kinases, on TNF-α-stimulated fractalkine expression by VSMCs. The effects of PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK on VSMC fractalkine gene expression were evaluated by incubating cells with various protein kinase inhibitors. Cells were first grown in DMEM containing 10% FCS until reaching 90% confluency. The medium was then replaced by DMEM containing 0.5% FCS for 24 h before treatment with a specific PKC inhibitor calphostin C (0.4 μM for 1 h), a selective PKA inhibitor H-89 (2 μM for 30 min), a p42/44 MAPK kinase inhibitor PD98059 (40 μM for 30 min), a p38 MAPK inhibitor SB203580 (40 μM for 30 min), or a PI-3K inhibitor wortmannin (0.5 μM for 30 min). After preincubation, cells were stimulated with TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. Further experiments were conducted to examine the role of NF-κB on TNF-α-stimulated fractalkine expression by VSMCs. The NF-κB inhibitors, MG132 (10 μM) and PDTC (200 μM) were incubated with VSMCs for 1.5 and 1 h, respectively. Then, cells were stimulated with TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. Additional studies were designed to examine the role of PTX in VSMC fractalkine expression. PTX (0.1–1 mg ml^{-1}) was first

incubated with VSMCs in the absence of TNF-α stimulation for 4 h at 37°C to determine the role of PTX on basal VSMC fractalkine expression. Further experiments were performed to examine the role of PTX on TNF-α-stimulated VSMC fractalkine expression. Cells were preincubated with PTX for 45 min, followed by TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. After incubation for the given periods, cell monolayers were washed and used for RNA (4-h stimulation) or protein (24-h stimulation) isolation as described below.

RT–PCR and Northern blot analysis

Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method (Chomczynski & Sacchi, 1987). Ten micrograms of total RNA were electrophoresed on formaldehyde-denatured 1% agarose gels and subsequently transferred to nylon membranes. cDNA fragments of rat fractalkine and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were first amplified by RT–PCR from glomerular RNA of Wistar rats using the following specific primer pairs: rat fractalkine, upstream 5′-attttccaagacagaggacc-3′, downstream 5′-gaagagtagaccaagaaagg-3′ (Harrison *et al.*, 1998), and rat GAPDH, upstream 5′-tcattgacctcaactacatg-3′, downstream 5′-caaagttgtcatggatgacc-3′ (Tso *et al.*, 1985). RT–PCR was performed by adding 3 μl (150 ng) of the native first-strand cDNA in a total of 10 μl containing 50 mM Tris, 0.25 mg ml^{-1} bovine serum albumin, 1 mM $MgCl_2$, 200 μM dNTPs, 33 pmoles of each primer, and 1 U DNA polymerase. The amplified products were eluted from polyacrylamide gels, and subcloned into pGEM-T vectors (Promega). The accuracy of the inserts were confirmed by DNA sequence analysis. The cloned cDNAs were linearized and used as templates for *in vitro* transcription of antisense digoxigenin-conjugated riboprobes, following the manufacturer's instructions (Roche Molecular Biochemicals). After hybridization, the blots were developed using CSPD® (Roche Molecular Biochemicals) as the substrate for alkaline phosphatase. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of GAPDH messages.

Western blot analysis

VSMCs were washed and lysed in RIPA buffer containing 1% IGEPAL CA-630 and 0.25% deoxycholate (Sigma). Forty micrograms of cell lysates were heated at 100°C for 10 min, applied to 7.5% (for cell-bound fractalkine) or 9% (for PKC, p42/44 MAPK, c-Jun, IκBα and β-actin) SDS-polyacrylamide gels, and electrophoresed. For detection of soluble fractalkine in the conditioned medium of TNF-α-activated VSMCs, media were concentrated with Centricon-10® (Millipore, Bedford, MA, U.S.A.), and 50 μg of protein were electrophoresed on 7.5% SDS-polyacrylamide gels. A prestained marker was also electrophoresed as a molecular weight marker. After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore) using a transblot chamber with Tris buffer. Western blots were incubated at 4°C overnight with primary antibodies. The next morning, membranes were washed with 1 × phosphate-buffered saline/5% Tween-20 at room temperature for 40 min, and incubated with peroxidase-conjugated second antibodies at room temperature for 1 h. After washing, the membranes were incubated with Renaissance® (NEN® Life Science, MA, U.S.A.) according to the manufacturer's instructions. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of β-actin wherever appropriate.

Immunocytochemistry

For demonstration of p65/NF-κB nuclear translocation, VSMCs were incubated with TNF-α (5 ng ml^{-1}), or vehicles for 7.5, 15, or 30 min before fixation with 4% paraformaldehyde for 1 h at 4°C. The cells were then washed by 1 × phosphate-buffered saline/0.2% TritonX-100 for 15 min and incubated with rabbit anti-p65/NF-κB at 4°C overnight. The next day, after washing for 15 min, the cells were incubated with biotin-conjugated anti-rabbit IgG at room temperature for 1 h. Then, the cells were washed and incubated with the avidin-biotin-peroxidase reagent (Dako, Glostrup, Denmark) at room temperature for 1 h. After washing, immunodetection for p65/NF-κB was performed by adding 3-amino-9-ethylcarbazole chromogen (Dako) as substrate according to the manufacturer's instructions.

Statistics

Data are expressed as mean ± s.e.mean. All comparisons were done by analysis of variance followed by Dunnett's *t*-test using the StatView® package for the Macintosh computer (Abacus Concepts, CA, U.S.A.). A probability value of less than 0.05 was considered statistically significant.

Results

Effects of TNF-α on fractalkine mRNA and protein expression

VSMCs were first incubated with different concentrations of TNF-α (1 to 50 ng ml^{-1}) for varying time periods (4 to 24 h). The Northern and Western blot results showed that at basal state VSMCs expressed a low level of a single 3.8-kb fractalkine mRNA species and a ~90-kDa cell-bound fractalkine protein. Exogenous TNF-α stimulated fractalkine mRNA and cell-bound protein expression in time- and dose-dependent manners (Figure 1). Further, our immunoblot results showed the presence of a ~70 kDa fractalkine polypeptide in the conditioned media of TNF-α-activated VSMCs (Figure 2). By using a broad-spectrum inhibitor of matrix metalloproteinases, GM 6001 (2 μM), we found that the amount of soluble fractalkine could be reduced by GM 6001, in association with an increase of the cell-bound form (Figure 2). These results indicate that TNF-α can stimulate VSMCs to produce cell-bound and soluble fractalkine, with the latter deriving from cleavage of the cell-bound form.

Signalling pathways mediating fractalkine expression by TNF-α

Because TNF-α activates multiple signalling pathways that include PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK (Vilcek & Lee, 1991; Heller & Kronke, 1994; Berghe *et al.*, 1998; Lallena *et al.*, 1999; Reddy *et al.*, 2000) experiments

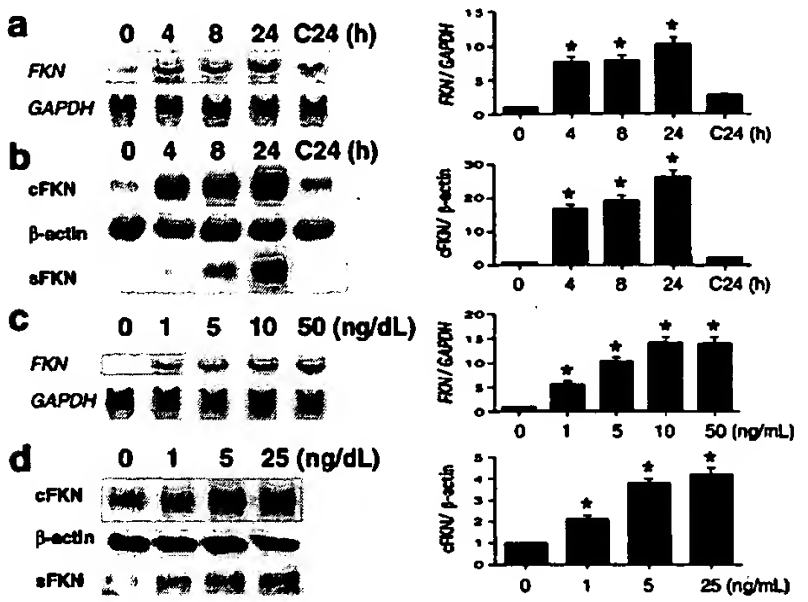


Figure 1 Time-course and dose-response of TNF-α stimulation on fractalkine mRNA and protein expression. VSMCs were incubated with TNF for the given periods. Ten micrograms of total RNA, forty micrograms of cell lysate, and fifty micrograms of concentrated condition media were analysed for fractalkine mRNA and protein expression as described in Methods. (a and b) Representative Northern and Western blots of fractalkine expression in response to TNF-α (5 ng ml^{-1}) at variable timepoints. *FKN*: fractalkine mRNA, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase mRNA, cFKN: cell-bound fractalkine in cell lysate, sFKN: soluble fractalkine in concentrated conditioned media. (c and d) Representative Northern and Western blots of fractalkine expression in response to TNF-α (1–50 ng ml^{-1}) at 4 and 24 h, respectively. Right panels show corresponding quantitative results of *FKN/GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs control at zero time.

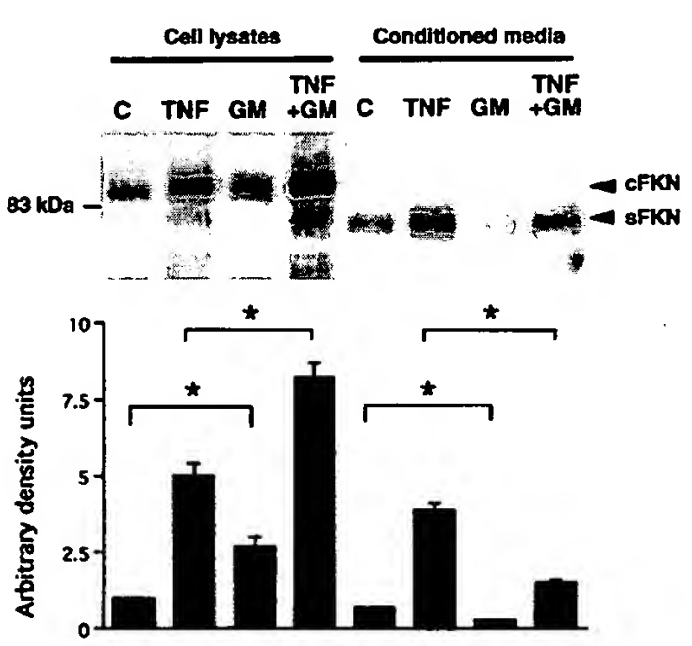


Figure 2 Effects of GM 6001 on TNF-α-stimulated cell-bound and soluble fractalkine expression. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 24 h, with or without pretreatment with GM (GM 6001, 2 μM) for 30 min. Upper panel shows representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panel shows quantitative results of cFKN (corrected for β-actin) and sFKN relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ between the given experimental conditions.

were performed to identify the role of these pathways on TNF-α-stimulated fractalkine expression. Our results indicated that the incubation of VSMCs with calphostin C (0.4 μM), a selective inhibitor of PKC, or PD98059 (40 μM), a specific inhibitor of p42/44 MAPK kinase, significantly attenuated TNF-α-stimulated VSMC fractalkine mRNA and protein expression (Figure 3). In contrast, H-89 (2 μM), a selective inhibitor of PKA, wortmannin (0.5 μM), a selective inhibitor of PI 3-K, and SB203580 (40 μM), a specific inhibitor of p38 MAPK did not have discernible effects on fractalkine expression (Figure 3).

Effects of NF-κB inhibition on TNF-α-stimulated fractalkine expression

The preincubation of cells with the ubiquitin/proteosome inhibitors, MG132 (Yamakawa *et al.*, 1999) and PDTC (Liu *et al.*, 1999) resulted in complete inhibition of TNF-α-stimulated fractalkine mRNA and protein expression (Figure 4). At the concentrations used in the present study, both MG132 and PDTC reversed TNF-α-induced degradation of I-κBα (Figure 5a,b) and nuclear translocation of p65/NF-κB subunit (Figure 6). Furthermore, MG132 and PDTC have been reported as activators for c-Jun/AP-1 pathway (Yakoo & Kitamura, 1996; Nakayama *et al.*, 2001) and our results showed that they did increase TNF-α-activated phospho-c-Jun levels (Figure 5a,b). In contrast, neither agent had discernible effects on the levels of TNF-α-stimulated phospho-PKC or phospho-44/42 MAPK (Figure 5a,b).

Additional experiments were performed to examine the effects of PD98059 and calphostin C on TNF-α-activated signalling cascades. Our results indicated that PD 98059, but not calphostin C, inhibited TNF-α-activated phospho-p42/44 MAPK and phospho-c-Jun levels whereas only calphostin C suppressed the levels of TNF-α-activated phospho-PKC (Figure 5c,d). Neither PD98059 nor calphostin C, however, affected TNF-α-induced degradation of I-κBα (Figure 5c,d) or nuclear translocation of p65/NF-κB (Figure 6).

Effects of PTX on TNF-α-stimulated fractalkine expression

The preincubation of VSMCs with PTX (0.1–1 mg ml^{-1}) alone had no effect on basal fractalkine mRNA or protein expression (data not shown). However, the pretreatment of VSMCs with PTX for 45 min dose-dependently attenuated TNF-α-stimulated fractalkine mRNA and protein expression (Figure 7a,b). Fractalkine transcript stability over time in cells incubated with or without PTX (1 mg ml^{-1}) is shown in Figure 8. Significant breakdown of fractalkine mRNA becomes detectable between 3 to 7 h after cessation of transcription by actinomycin D (20 μg ml^{-1}) in the control cells. In cells treated with PTX, no difference in the rate of fractalkine mRNA breakdown was apparent from control; a clear reduction in the number of fractalkine mRNA transcripts in the presence of PTX also occurs between 3 to 7 h. These results suggest that PTX reduced TNF-α-stimulated fractalkine mRNA levels *via* inhibition of transcription, rather than *via* enhancement of mRNA transcript breakdown.

To further explore the underlying mechanisms, PTX-pretreated VSMCs were stimulated with TNF-α (5 ng ml^{-1}) for 7.5 to 30 min. The results showed that PTX attenuated TNF-α-activated phosphorylation of PKC and p42/44 MAPK, and prevented TNF-α-induced degradation of I-

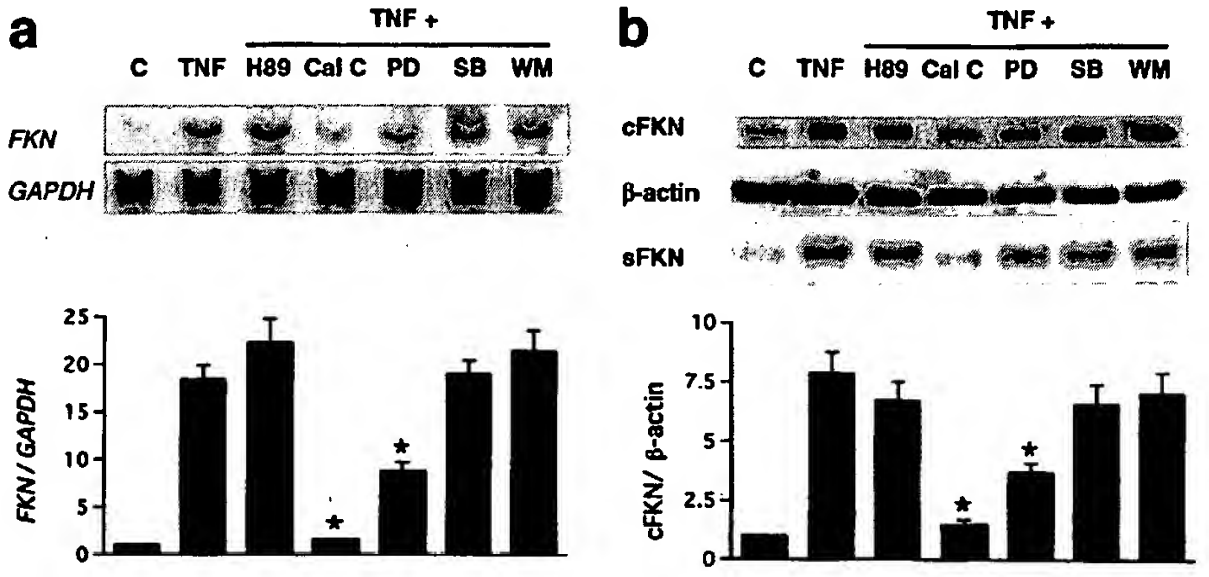


Figure 3 Effects of protein kinase inhibitors on TNF-α stimulated fractalkine mRNA and protein expression. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 4 or 24 h, with or without pretreatment with the given pharmacologic inhibitors (H-89, 2 μM; Cal C: calphostin C, 0.4 μM; PD: PD98059, 40 μM; SB: SB203580, 40 μM; WM: wortmannin, 0.5 μM). (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN/GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells.

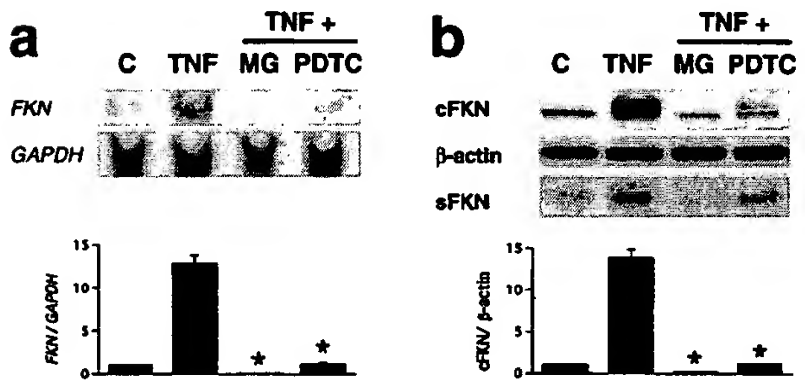


Figure 4 Effect of NF-κB inhibition on TNF-α stimulated fractalkine mRNA and protein expression. VSMCs were pretreated with MG (MG132, 10 μM) or PDTC (pyrrolidine dithiocarbamate, 200 μM) for 1.5 and 1 h, respectively, followed by TNF-α (5 ng ml^{-1}) for 4 or 24 h. (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN/GAPDH* and cFKN/β-actin ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells.

κBα (Figure 7c) and nuclear translocation of p65/NF-κB (Figure 6). In addition, PTX attenuated phosphorylation of c-Jun activated by TNF-α (Figure 7c).

Discussion

The present study shows that cultured VSMCs at steady-state express a low level of fractalkine mRNA and protein, which could be up-regulated by TNF-α in both time- and dose-dependent manners. In addition to cell-bound fractalkine, this study shows that the conditioned media of TNF-α-activated VSMC contain a ~75-kD soluble fractalkine, which is likely shed from the cell-bound fractalkine. This finding is consistent with recent observations that cleavage of the cell-bound form by metalloproteinases is the major source for soluble fractalkine in various cultured cell systems, including VSMCs (Chapman *et al.*, 2000a; Garton *et al.*, 2001; Ludwig *et al.*, 2002). The biological significance of the cleaved soluble form of fractalkine remains controversial. While the soluble fractalkine was originally described as a novel chemoattractant (Bazan *et al.*, 1997), recent *in vitro* studies performed in cultured endothelial cells and VSMCs fail to support such a notion (Ludwig *et al.*, 2002; Chapman *et al.*, 2000b; Umehara *et al.*, 2001b). On the other hand, in the central nervous system soluble fractalkine shed from neurons has been reported to act as a chemoattractant for monocytes, or as a neurotransmitter for microglial cells (Chapman *et al.*, 2000a; Harrison *et al.*, 1998; Tong *et al.*, 2000).

The signal pathways initiated by TNF-α include those that activate protein kinases such as PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK (Vilcek & Lee, 1991; Heller & Kronke, 1994; Berghe *et al.*, 1998; Lallena *et al.*, 1999; Reddy *et al.*, 2000), and transcription factors such as NF-κB and AP-1 (Heller & Kronke, 1994; Malinin *et al.*, 1997). This study shows that TNF-α-stimulated VSMC fractalkine mRNA and protein expression is attenuated by pharmacologic inhibitors of PKC (calphostin C) and p42/44 MAPK kinase (PD98059), but not PKA (H-89), PI-3K (wortmannin), or p38 MAPK (SB203580), indicating that the intracellular signals mediating TNF-α-stimulated fractalkine expression involve activation of PKC and p42/44 MAPK, rather than PKA, PI 3-K, or p38 MAPK pathways.

NF-κB is a key nuclear factor regulating the transactivation of genes involved in a variety of chronic inflammatory disorders, including atherosclerosis (Barnes & Karin, 1997; Bourcier *et al.*, 1997; Brand *et al.*, 1997). This study shows that inhibition of the NF-κB signal by the ubiquitin/proteosome inhibitors, MG132 and PDTC, abolished TNF-α-stimulated VSMC fractalkine mRNA and protein expression, indicating that NF-κB plays a crucial role in fractalkine gene transcription in VSMCs. Parallel to this finding, a recent study shows that fractalkine induction in rat aortic endothelial cells by inflammatory cytokines is NF-κB-dependent (Garcia *et al.*, 2000).

In this study, we show that suppression of the classical/novel and atypical PKC pathways by calphostin C causes an inhibition in TNF-α-stimulated fractalkine gene expression at a concentration that did not affect nuclear translocation of p65/NF-κB. This suggests that mechanisms for the anti-fractalkine effect of calphostin C is either beyond p65/NF-κB nuclear translocation or through another transcription factor. Consistent with this notion, PKCζ has been shown to positively regulate κB-dependent transcription activity *via* direct phosphorylation of the transactivation domain of p65/NF-κB (Anrather *et al.*, 1999). Thus, it is possible that inhibition of TNF-α-activated PKCζ by calphostin C may directly down-regulate the transactivation potential of NF-κB in the nucleus without involvement of IκB modulation. Similarly, PD98059 was found to inhibit TNF-α-stimulated fractalkine gene expression at a concentration that did not affect nuclear translocation of p65/NF-κB. Inhibition of p42/44 MAPK kinase by PD98058 has been reported to directly suppress nuclear p65/NF-κB transactivation potential without IκB regulation (Berghe *et al.*, 1998), and overexpression of a dominant negative p42/44 MAPK mutant has been shown to block κB-dependent promotor activation by TNF-α, in association with a reduction in AP-1 but not NF-κB nuclear levels (Berra *et al.*, 1995). Because c-Jun/AP-1 protein has been shown to mediate TNF-α-activated gene expression *via* interaction with p65/NF-κB (Stein *et al.*, 1993; Ahmad *et al.*, 1998; Kyriakis, 1999), we speculate that down-regulation of the c-Jun/AP-1 pathway by PD98059 may attenuate the transactivation potential of nuclear NF-κB. Our result that PD98059 decreases phospho-c-Jun levels induced by TNF-α was consistent with such a possibility. While AP-1 may synergize with NF-κB for fractalkine gene transcription, activation of c-Jun/AP-1 alone, without NF-κB, may not be sufficient for fractalkine gene expression. This notion is supported at least partially by the present data that MG132 and PDTC completely blocks TNF-α-stimulated fractalkine mRNA expression despite the presence of an augmented phospho-c-Jun level.

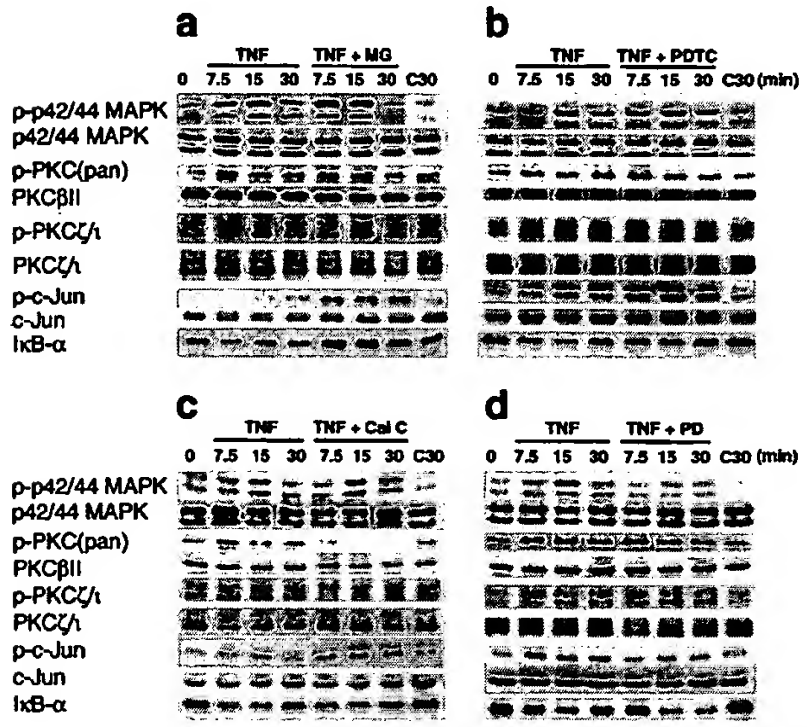


Figure 5 Effects of MG132, PDTC, calphostin C, and PD98059 on TNF-α-activated phospho-p42/44 MAPK, phospho-PKC, phospho-c-Jun and I-κBα levels. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for varying timepoints, with or without pretreatment with the given pharmacologic inhibitors. Graphs showing representative Western blots from three independent experiments with similar results. The concentrations of inhibitors used in the signalling studies were MG: MG132 (10 μM); PDTC: pyrrolidine dithiocarbamate (200 μM); Cal C: calphostin C (0.4 μM); PD: PD98059 (40 μM). PKC(pan): PKC of classical/novel isoforms; PKCζ/ι: PKC of atypical isoformζ/ι.

This study shows that PTX decreases the levels of TNF-α-stimulated fractalkine mRNA transcripts, which are thus unavailable for translation into protein. Based on the present study on fractalkine-RNA transcript stability, PTX appears to act by inhibiting TNF-α-augmented fractalkine transcription rather than enhancing fractalkine-mRNA breakdown. Furthermore, our results show that PTX blocks TNF-α-induced I-κBα degradation and p65/NF-κB nuclear translocation, indicating that PTX may suppress TNF-α-stimulated

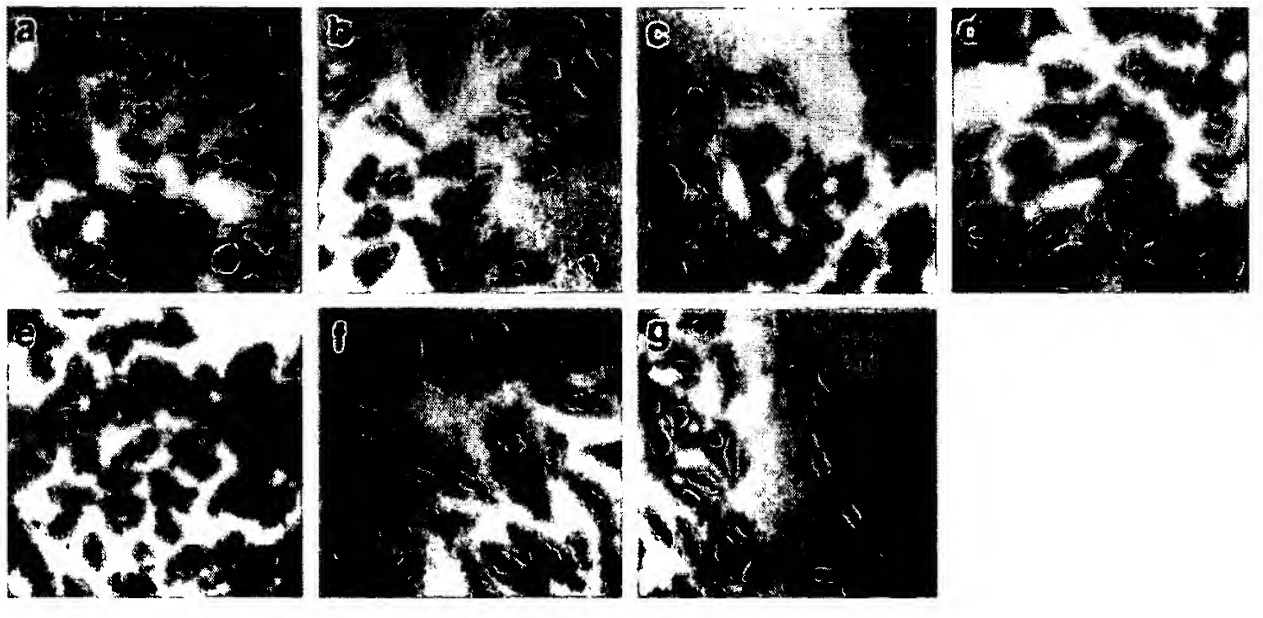


Figure 6 Immunostaining of nuclear translocated NF-κB/p65 induced by TNF-α: effects of MG132, PDTC, and pentoxifylline. (a) control. (b) incubation of VSMCs with TNF-α (5 ng ml^{-1}) for 15 min induced nuclear translocation of p65/NF-κB. (c, d, e) MG132 (10 μM), pyrrolidine dithiocarbamate (200 μM), and pentoxifylline (1 mg ml^{-1}) blocked translocation of p65/NF-κB induced by TNF-α. (f, g) Calphostin C (0.4 μM) and PD98059 (40 μM) did not affect NF-κB/p65 nuclear translocation (original magnification × 300).

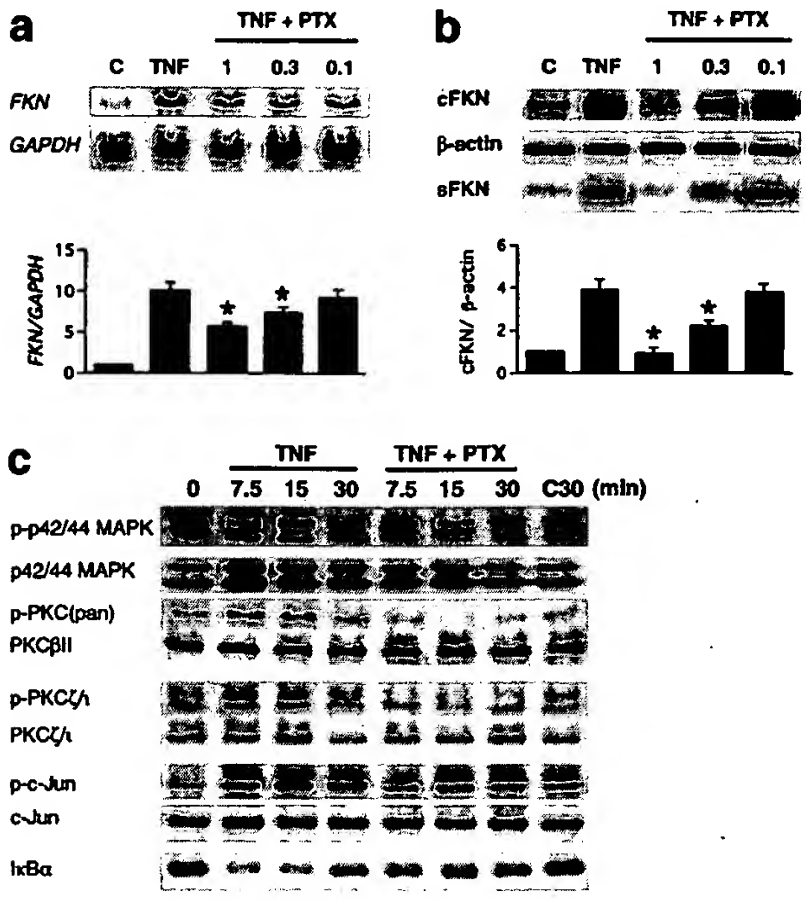


Figure 7 Effects of pentoxifylline on TNF-α stimulated fractalkine mRNA and protein expression as well as TNF-α-activated phospho-p42/44 MAPK, phospho-PKC, phospho-c-Jun and I-κBα levels. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 4 or 24 h, with or without pretreatment with PTX (pentoxifylline, 1–0.1 mg ml^{-1}) for 45 min. (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN/GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells. (c) Representative Western blots showing the effects of PTX (1 mg ml^{-1}) on TNF-α-activated signalling pathways. These experiments were performed three times, and similar results were obtained. PKC(pan): PKC of classical/novel isoforms; PKCζ/ι: PKC of atypical isoformζ/ι.

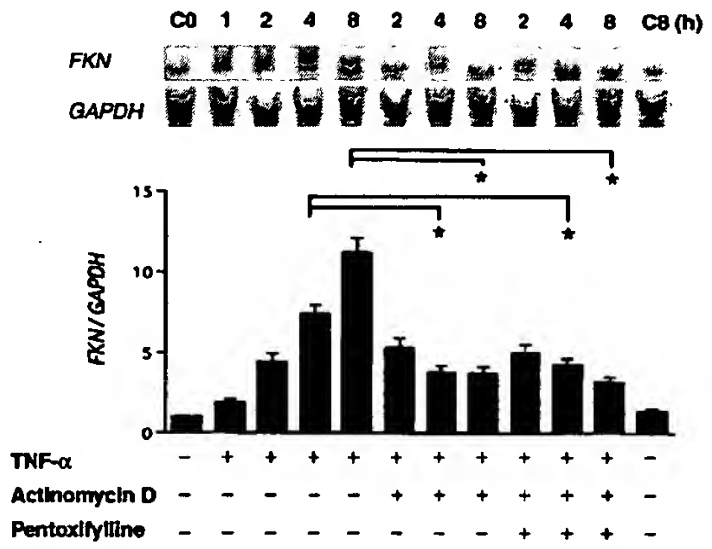


Figure 8 Northern blot of TNF-α-stimulated fractalkine mRNA stability. Cells were exposed to TNF-α (5 ng ml^{-1}) for 1 h, followed by actinomycin D (20 μg ml^{-1}) with or without pentoxifylline (1 mg ml^{-1}) to stop further mRNA transcription. Cells were harvested at serial timepoints as indicated to assess the rate of fractalkine mRNA breakdown. Upper panel shows representative Northern blots. Significant fractalkine mRNA transcript breakdown appears to occur between 3 to 7 h after addition of actinomycin D. Lower panel shows quantitative results of *FKN/GAPDH* mRNA ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs corresponding TNF-α-treated cells.

fractalkine gene transcription *via* down-regulation of NF-κB activation. The ability of PTX to antagonize NF-κB activity in VSMCs has been reported elsewhere, but the exact mechanism remains poorly elucidated (Bellas *et al.*, 1995; Bretschneider *et al.*, 1997). As demonstrated by this study, the anti-NF-κB activity of PTX is mediated, at least in part, by cytoplasmic retention of I-κBα-p65 complexes. Because PTX is an inhibitor of cyclic 3′,5′-nucleotide phosphodiesterase (Chen *et al.*, 1999), one would speculate that this effect is initiated by activation of the cAMP-PKA cascade, which in turn suppresses the *raf*-1/ p42/44 MAPK pathway (Pinzani *et al.*, 1996; D'Angelo *et al.*, 1997) and the resultant activation of NF-κB (Neumann *et al.*, 1995). Consistent with this notion, our results show that PTX decreases TNF-α-activated phospho-p42/44 MAPK levels. On the other hand, Lee *et al.* (1997) have reported that the anti-NF-κB activity of PTX is not mediated by phosphodiesterase inhibition, and Biswas *et al.* (1994) have shown that PTX inhibits NF-κB activation *via* a PKC-, but not PKA-dependent mechanism. Thus, the anti-NF-κB activity of PTX may not be cAMP-PKA-dependent. In this study, we found that PTX reduced TNF-α-induced phospho-PKC and phospho-c-Jun levels, suggesting that PTX may also modulate NF-κB activity in a way similar to calphostin C and PD98059.

In conclusion, we have demonstrated that TNF-α stimulates fractalkine gene and protein expression in rat VSMCs, and blockade of PKC, p42/44 MAPK kinase, and NF-κB nuclear translocation correlates with inhibition of fractalkine gene and protein expression. In view of the emerging importance of fractalkine in the recruitment of mononuclear leukocytes, these data may open possibilities for designing novel interventions or for using currently available agents to modulate the inflammatory response within the vessel wall. One example presented in this study is suppression of TNF-α-stimulated fractalkine gene and protein expression by the haemorrheologic agent PTX through down-regulation of the PKC, p42/44 MAPK, and p65/NF-κB pathways.

This work was supported by grants from the National Science Council, 89-2314-B002-068, the Ta-Tung Kidney Foundation, and the Mrs Hsiu-Chin Lee Kidney Research Fund, Taipei, Taiwan.

References

AHMAD, M., THEOFANIDIS, P. & MEDFORD, R.M. (1998). Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-α. *J. Biol. Chem.*, **273**, 4616–4621.

ANRATHER, J., CSIZMADIA, V., SOARES, M.P. & WINKLER, H. (1999). Regulation of NF-κB relA phosphorylation and transcriptional activity by p21ras and protein kinase Cζ in primary endothelial cells. *J. Biol. Chem.*, **274**, 13594–13603.

BARNES, P.J. & KARIN, S. (1997). Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.*, **336**, 1066–1071.

BAZAN, J.F., BACON, K.B., HARDIMAN, G., WANG, W., SOO, K., ROSS, D., GREAVES, D.R., ZLOTNIK, A. & SCHALL, T.J. (1997). A new class of membrane-bound chemokine with a CX_3C motif. *Nature*, **385**, 640–644.

BELLAS, R.E., LEE, J.S. & SONENSHEIN, G.E. (1995). Expression of a constitutive NF-κB-like activity is essential for proliferation of cultured bovine vascular smooth muscle cells. *J. Clin. Invest.*, **96**, 2521–2527.

BERGHE, W.V., PLAISANCE, S., BOONE, E., DE BOSSCHER, K., SCHMITZ, M.L., FIERS, W. & HAEGEMAN, G. (1998). p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.*, **273**, 3285–3290.

BERRA, E., DIAZ-MECO, M.T., LOZANO, J., FRUTOS, S., MUNICIO, M.M., SANCHEZ, P., SANZ, L. & MUSCAT, J. (1995). Evidence for a role of MEK and MAPK during signal transduction by protein kinase zeta. *EMBO J.*, **14**, 6157–6163.

BISWAS, D.K., AHLERS, C.M., DEZUBE, B.J. & PARDEE, A.B. (1994). Pentoxifylline and other protein kinase inhibitors down-regulate HIV-LTR NF-κB induced gene expression. *Mol. Med.*, **1**, 31–43.

BOURCIER, T., SUKHOVA, G. & LIBBY, P. (1997). The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J. Biol. Chem.*, **272**, 15817–15824.

BRAND, K., PAGE, S., WALLI, A.K., NEUMEIER, D. & BAEUERLE, P.A. (1997). Role of nuclear factor-kappa B in atherogenesis. *Exp. Physiol.*, **82**, 297–304.

BRAUN, M., PIETSCH, P., SCHROR, K., BAUMANN, G. & FELIX, S.B. (1999). Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovasc. Res.*, **41**, 395–401.

BRETSCHNEIDER, E., WITTOPTH, M., WEBER, A.A., GLUSA, E. & SCHROR, K. (1997). Activation of NfkappaB is essential but not sufficient to stimulate mitogenesis of vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **235**, 365–368.

CHAPMAN, G.A., MOORES, K., HARRISON, D., CAMPBELL, C.A., STEWART, B.R. & STRIJBOS, P.J.L.M. (2000a). Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J. Neurosci.*, **20**, RC87 (1-5).

CHAPMAN, G.A., MOORES, K.E., GOHIL, J., BERKHOUT, T.A., PATEL, L., GREEN, P., MACPHEE, C.H. & STEWART, B.R. (2000b). The role of fractalkine in the recruitment of monocytes to the endothelium. *Eur. J. Pharmacol.*, **392**, 189–195.

CHEN, Y-M., WU, K-D., TSAI, T-J. & HSIEH, B-S. (1999). Pentoxifylline inhibits PDGF-induced proliferation of and TGF-β-stimulated collagen synthesis by vascular smooth muscle cells. *J. Mol. Cell. Cardiol.*, **31**, 773–783.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single step method for RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **163**, 156–162.

D'ANGELO, G., LEE, H. & WEINER, R.I. (1997). cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. *J. Cell Biochem.*, **67**, 353–366.

FONG, A.M., ROBINSON, L.A., STEEBER, D.A., TEDDER, T.F., YOSHIE, O., IMAI, T. & PATEL, D.D. (1998). Fractalkine and CX_3C R1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J. Exp. Med.*, **188**, 1413–1419.

GARCIA, G.E., XIA, Y., CHEN, S., WANG, Y., YE, R.D., HARRISON, J.K., BACON, K.B., ZERWES, H-G. & FENG, L. (2000). NF-κB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1β, TNF-α, and LPS. *J. Leukoc. Biol.*, **67**, 577–584.

GARTON, K.J., GOUGH, P.J., BLOBE, C.P., MURPHY, G., GREAVES, D.R., DEMPSEY, P.J. & RAINES, E.W. (2001). Tumor necrosis factor-α-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX_3CL1). *J. Biol. Chem.*, **276**, 37993–38001.

GERSZTEN, R.E., MACH, F., SAUTY, A., ROSENZWEIG, A. & LUSTER, A.D. (2000). Chemokines, leukocytes, and atherosclerosis. *J. Lab. Clin. Med.*, **136**, 87–92.

GREAVES, D.R., HAKKINEN, T., LUCAS, A.D., LIDDIARD, K., JONES, E., QUINN, C.M., SENARATNE, J., GREEN, F.R., TYSON, K., BOYLE, J., SHANAHAN, C., WEISSBERG, P.L., GORDON, S. & YLA-HERTUALLA, S. (2001). Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine and thymus- and activation-regulated chemokine, are expressed in human arteriosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.*, **21**, 923–929.

HARRISON, J.K., JIANG, Y., CHEN, S., XIA, Y., MACIEJEWSKI, D., MCNAMARA, R.K., STREIT, W., SALAFRANCA, M.N., ADHIKARI, S., THOMPSON, D.A., BOTTI, P., BACON, K.B. & FENG, L. (1998). Role of neuronally derived fractalkine in mediating interaction between neurons and CX_3CR1-expressing microglia. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 10896–10901.

HELLER, R.A. & KRONKE, M. (1994). Tumor necrosis factor receptor-mediated signaling pathways. *J. Cell Biol.*, **126**, 5–9.

IMAI, T., HIESHIMA, K., HASKELL, C., BABA, M., NAGIRA, M., NISHIMURA, M., KAKIZAKI, M., TAKAGI, S., NOMIYAMA, H., SCHALL, T.J. & YOSHIE, O. (1997). Identification and molecular characterization of fractalkine receptor CX_3C R1, which mediates both leukocyte migration and adhesion. *Cell*, **91**, 521–530.

KANAZAWA, N., NAKAMURA, T., TASHIRO, K., MURAMATSU, M., MORITA, K., YONEDA, K., INABA, K., IMAMURA, S. & HONJO, T. (1999). Fractalkine and macrophage-derived chemokine: T cell-attracting chemokines expressed in T cell area dendritic cells. *Eur. J. Immunol.*, **29**, 1925–1932.

KYRIAKIS, J.M. (1999). Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Exp.*, **7**, 217–231.

LALLENA, M.J., DIAZ-MECO, M.T., BREN, G., PAYA, C.V. & MOSCAT, J. (1999). Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol. Cell. Biol.*, **19**, 2180–2188.

LEE, K.S., COTTAM, H.B., HOUGLUM, K., WASSON, D.B., CARSON, D. & CHOJKIER, M. (1997). Pentoxifylline blocks hepatic stellate cell activation independently of phosphodiesterase inhibitory activity. *Am. J. Physiol.*, **273**, G1094–G1100.

LIU, S.F., YE, X. & MALIK, A.B. (1999). Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. *Circulation*, **100**, 1330–1337.

LUCAS, A., CHADWICK, N., WARREN, B.F., JEWELL, D.P., GORDON, S., POWRIE, F. & GREAVES, D.R. (2001). The transmembrane form of the CX_3CL1 chemokine fractalkine is expressed predominantly by epithelial cells *in vivo*. *Am. J. Pathol.*, **158**, 855–866.

LUDWIG, A., BERKHOUT, T., MOORES, K., GROOT, P. & CHAPMAN, G. (2002). Fractalkine is expressed by smooth muscle cells in response to IFN-γ and TNF-α and is modulated by metalloproteinase activity. *J. Immunol.*, **168**, 604–612.

MALININ, N.L., BOLDIN, M.P., KOVALENKO, A.V. & WALLACH, D. (1997). MAP3K-related kinase involved in NF-κB induction by TNF, CD95, and IL-1. *Nature*, **385**, 540–544.

MCDERMOTT, D.H., HALCOX, J.P.J., SCHENKE, W.H., WACLAWIW, M.A., MERRELL, M.N., EPSTEIN, N., QUYYUMI, A.A. & MURPHY, P.M. (2001). Association between polymorphism in the chemokine receptor CX_3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. *Circ. Res.*, **89**, 401–407.

MOATTI, D., FAURE, S., FUMERON, F., AMARA, M.E.W., SEKNADJI, P., MCDERMOTT, D.H., DEBRE, P., AUMONT, M.C., MURPHY, P.M., DE PROST, D. & COMBADIERE, C. (2001). Polymorphism in the fractalkine receptor CX_3CR1 as a genetic risk factor for coronary artery disease. *Blood*, **97**, 1925–1928.

NAKAYAMA, K., FURUSU, A., XU, Q., KONTA, T. & KITAMURA, M. (2001). Unexpected transcriptional induction of monocyte chemoattractant protein 1 by proteosome inhibition: Involvement of the c-Jun N-terminal kinase-activator protein 1 pathway. *J. Immunol.*, **167**, 1145–1150.

NEUMANN, M., GRIESHAMMER, T., CHUVPILO, S., KNEITZ, B., LOHOFF, M., SCHIMPL, A., FRANK, JR, B.R. & SERFLING, E. (1995). RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. *EMBO J.*, **14**, 1991–2004.

PAPADOPOULOS, E.J., SASSETTI, C., SAEKI, H., YAMADA, N., KAWAMURA, T., FITZHUGH, D.J., SARAF, M.A., SCHALL, T., BLAUVELT, A., ROSEN, S.D. & HUANG, S.T. (1999). Fractalkine, a CX_3C chemokine, is expressed by dendritic cells and is up-regulated upon dendritic cell maturation. *Eur. J. Immunol.*, **29**, 2551–2559.

PINZANI, M., MARRA, F., CALIGIURI, A., DEFRANCO, R., GENTILINI, A., FAILLI, P. & GENTILINI, P. (1996). Inhibition by pentoxifylline of extracellular signal-regulated kinase activation by platelet-derived growth factor in hepatic stellate cells. *Br. J. Pharmacol.*, **119**, 1117–1124.

PRICE, D. & LOSCALZO, J. (1999). Cellular adhesion molecules and atherosclerosis. *Am. J. Med.*, **107**, 85–97.

REAPE, T.J. & GROOT, P.H.E. (1999). Chemokines and atherosclerosis. *Atherosclerosis*, **147**, 213–225.

REDDY, S.A., HUANG, J.H. & LIAO, W.S. (2000). Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappa B activation. *J. Immunol.*, **164**, 1355–1363.

ROSS, R. (1999). Atherosclerosis-an inflammatory disease. *N. Engl. J. Med.*, **340**, 115–126.

SASAYAMA, S., OKADA, M. & MATAUMORI, A. (2000). Chemokines and cardiovascular diseases. *Cardiovasc. Res.*, **45**, 267–269.

SCHWAEBLE, W.J., STOVER, C.M., SCHALL, T.J., DAIRAGHI, D.J., TRINDER, P.K., YOSHIDA, H., IMAIZUMI, T., FUJIMOTO, K., MATSUO, N., KIMURA, K., CUI, X-F., MATSUMIYA, T., TANJI, K., SHIBATA, T., TAMO, W., KUMAGAI, M. & SATOH, K. (2001). Synergistic stimulation, by tumor necrosis factor-α and interferon-γ, of fractalkine expression in human astrocytes. *Neurosci. Lett.*, **303**, 132–136.

STEIN, B., BALDWIN, JR, A.S. BALLARD, D.W., GREENE, W.C., ANGEL, P. & HERRLICH, P. (1993). Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potential biological function. *EMBO J.*, **12**, 3879–3891.

TONG, N., PERRY, S.W., ZHANG, Q., JAMES, H.J., GUO, H., BROOKS, A., BAL, H., KINNEAR, S.A., FINE, S., EPSTEIN, L.G., DAIRAGHI, D., SCHALL, T.J., GENDELMAN, H.E., DEWHURST, S., SHARER, L.R. & GELBARD, H.A. (2000). Neuronal fractalkine expression in HIV-1 encephalitis. Roles for macrophage recruitment and neuroprotection in the central nervous system. *J. Immunol.*, **164**, 1333–1339.

TSO, J-Y., SUN, X-H., KAO, T-H., REECE, K.S. & WU, R. (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.*, **13**, 2485–2502.

UMEHARA, H., BLOOM, E.T., OKAZAKI, T., DOMAE, N. & IMAI, T. (2001a). Fractalkine and vascular injury. *Trends Immunol.*, **22**, 602–607.

UMEHARA, H., GODA, S., IMAI, T., NAGANO, Y., MINAMI, Y., TANAKA, Y., OKAZAKI, T., BLOOM, E.T. & DOMAE, N. (2001b). Fractalkine, a CX_3C-chemokine, functions predominantly as an adhesion molecule in monocytic cell line THP-1. *Immunol. Cell. Biol.*, **79**, 298–302.

VILCEK, J. & LEE, T.H. (1991). Tumor necrosis factor: new insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.*, **266**, 7313–7316.

YAKOO, T. & KITAMURA, M. (1996). Antioxidant PDTC induces stromelyin expression in mesangial cells via a tyrosine kinase-AP-1 pathway. *Am. J. Physiol.*, **270**, F806–F811.

YAMAKAWA, T., EQUCHI, S., MATSUMOTO, T., YAMAKAWA, Y., NUMAGUCHI, K., MIYATA, I., REYNOLD, C.M., MOTLEY, E.D. & INAGAMI, T. (1999). Intracellular signaling in rat cultured vascular smooth muscle cells: roles of nuclear factor-kappaB and p38 mitogen-activated protein kinases on tumor necrosis factor-alpha production. *Endocrinology*, **140**, 3562–3572.

(Received August 19, 2002
Revised October 31, 2002
Accepted November 6, 2002)

Pentoxifylline blocks hepatic stellate cell activation independently of phosphodiesterase inhibitory activity

KWAN S. LEE,[1,2] HOWARD B. COTTAM,[3] KARL HOUGLUM,[1]
D. BRUCE WASSON,[3] DENNIS CARSON,[3] AND MARIO CHOJKIER[1]
[1]*Department of Medicine, Veterans Affairs Medical Center, Center for Molecular Genetics;*
[3]*The Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, California 92161; and* [2]*Department of Internal Medicine, Yonsei University College of Medicine, Seoul, South Korea 135-270*

Lee, Kwan S., Howard B. Cottam, Karl Houglum, D. Bruce Wasson, Dennis Carson, and Mario Chojkier. Pentoxifylline blocks hepatic stellate cell activation independently of phosphodiesterase inhibitory activity. *Am. J. Physiol.* 273 (*Gastrointest. Liver Physiol.* 36): G1094–G1100, 1997.— Activated, but not quiescent, hepatic stellate cells (lipocytes) have a high level of collagen type I and smooth muscle actin (SMA) gene expression. Therefore, stellate cell activation is a critical step in hepatic fibrosis. The mechanisms leading to stellate cell activation in vivo are unknown. The characteristic hepatic oxidative stress cascade induced in rats by CCl_4 markedly stimulated stellate cell entry into S phase, nuclear factor (NF)-κB activity, and c-*myb* expression. These changes were prevented by pentoxifylline, which also decreased CCl_4-induced hepatic injury. As expected, cAMP-mediated phosphorylation of CREB-Ser[133] was induced in vivo in stellate cells by pentoxifylline but not by its metabolite 5, an *N*-1 carboxypropyl derivative, which lacks phosphodiesterase inhibitory activity. Stellate cell nuclear extracts from CCl_4-treated, but not from control, animals formed a complex with the critical promoter E box of the α-SMA gene, which was disrupted by c-*myb* antibodies and competed with by c-*myb* cognate DNA. Treatment with pentoxifylline or metabolite 5 prevented the molecular abnormalities characteristic of stellate cell activation induced by CCl_4. These results suggest that induction of c-*myb* plays an important role in the in vivo activation of stellate cells. Pentoxifylline blocks stellate cell activation in vivo independently of its inhibitory effects on phosphodiesterases by interfering with the oxidative stress cascade and the activation of NF-κB and c-*myb*.

CREB phosphorylation; liver fibrogenesis; c-*myb* expression

OVERPRODUCTION OF COLLAGEN type I by activated hepatic stellate cells is a critical step in the development of liver cirrhosis (14–16, 26). However, the mechanisms leading to stellate cell activation in vivo remain unclear, and there is no established treatment for hepatic fibrogenesis (9).

Pentoxifylline is an alkylated xanthine, which is clinically useful for the treatment of conditions involving defective regional microcirculation (38). Although pentoxifylline, a nonspecific inhibitor of cyclic nucleotide phosphodiesterases (28), inhibits collagen gene expression in dermal fibroblasts (4), the mechanisms responsible for this effect are unknown. Moreover, the mechanisms responsible for the prevention of hepatic fibrosis by pentoxifylline in yellow phosphorus-induced hepatocellular necrosis (29) remain to be determined.

In this context, oxidative stress appears to play an essential role, through the induction of c-*myb* and nuclear factor (NF)-κB, in the activation of cultured stellate cells by collagen type I matrix or transforming growth factor (TGF)-α (24), suggesting that a similar mechanism may be responsible for stellate cell activation in vivo. Pentoxifylline inhibits NF-κB activity, resulting in a decreased expression of the human immunodeficiency virus-1 long terminal repeat (5), and prevents the lipopolysaccharide-induced production of tumor necrosis factor (TNF)-α (25, 32), which in turn activates NF-κB (18). Because NF-κB activity is induced by oxidative stress signals (18, 24), resulting in stellate cell activation (24), we tested whether pentoxifylline prevents the in vivo activation of stellate cells.

In this study, we analyzed the mechanisms leading to stellate cell activation in vivo in hepatic injury induced by CCl_4 (3, 31). We found that oxidative stress results in enhanced NF-κB and c-*myb* binding activities, which appear to be critical in stellate cell activation, and that pentoxifylline blocks these molecular events as well as stellate cell activation. Moreover, the metabolite 5 of pentoxifylline, an *N*-1 carboxypropyl derivative (12, 38) that lacks phosphodiesterase inhibitory activity, also prevented the activation of stellate cells. These findings suggest that the inhibition of stellate cell activation by pentoxifylline results from blocking the oxidative stress cascade within stellate cells, rather than from its inhibition of phosphodiesterases.

METHODS

Animals. Sprague-Dawley male rats (50–60 g) each received a single intraperitoneal injection of CCl_4 in mineral oil (1:3, vol/vol) at a dose of 2 ml/kg body wt (3) (CCl_4, pentoxifylline, and metabolite 5 groups) or mineral oil only (control group). In addition, animals received intraperitoneal injections (100 μl) of saline (control and CCl_4 groups), 200 mg/kg pentoxifylline (pentoxifylline group), or 200 mg/kg of the ester prodrug of pentoxifylline metabolite 5 (metabolite 5 group) at the following times with respect to mineral oil or CCl_4 administration: −4 h, +8 h, +20 h, +32 h, and +44 h. The last injection (at +44 h) included 30 μCi of 6-[^{3}H]thymidine (DuPont). Forty-eight hours after the CCl_4 or mineral oil injection (and 4 h after they received [^{3}H]thymidine), animals were killed and liver tissues were promptly removed, and a piece was fixed in 10% formaldehyde and embedded in paraffin for immunohistochemical staining (3). In some studies, blood samples were obtained at 24, 36, and 48 h after the CCl_4 or mineral oil injection.

Cell isolation. Stellate cells were prepared from rats of the experimental groups described above by in situ perfusion and

single-step density Nycodenz gradient (Accurate Chemical & Scientific, Westbury, NY), as described previously (3, 19, 24). Cells were mixed with 9.5 ml Hanks' containing 3 g/l bovine serum albumin and 8 ml of 28.7% (wt/vol) Nycodenz in sodium-free Hanks' buffer. The gradient was generated by placing 6 ml of Hanks' albumin solution on top of the liver cell mixture in a 50-ml centrifugation tube. After centrifugation (1,000 *g*, 4°C, 20 min), cells were aspirated from above the interface, washed twice in serum-free Dulbecco's modified Eagle's regular glucose medium, and collected. Stellate cells were identified by their typical autofluorescence at 328-nm excitation wavelength, staining of lipid droplets by oil red, and immunohistochemistry with a monoclonal antibody against desmin (3). Greater than 95% of the isolated cells were stellate cells (3, 19, 24). In some experiments, cells were cultured on a collagen type I matrix as described previously (24) and treated with pentoxifylline (100 μM) or metabolite 5 (100 μM) every day for 6 days. Cells were labeled with 2 μCi [^{3}H]thymidine (70–80 Ci/mmol, Amersham). After 3 h of labeling, cells were harvested and [^{3}H]thymidine incorporation into DNA was determined as described (8). Detection of α-SMA and cAMP-responsive element binding protein (CREB)-PSer^{133} in stellate cell extracts was performed by Western blot as described (8, 36) and using antibodies directed against α-SMA (Vector Laboratories) or CREB-PSer^{133} (Upstate Biotechnology).

NIH/3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (34) and labeled with [^{3}H]thymidine as described for stellate cells.

Nuclear extract preparation. Nuclei were prepared by a modification of the procedure described previously (7, 8, 24). Cells were homogenized in 1 ml of 5% citric acid, 0.5% Nonidet P-40, 10 mM NaF, and 10 mM Na pyrophosphate with a glass Dounce homogenizer with a loose-fitting pestle. The homogenized cells were placed above a cushion consisting of 30% sucrose and 1% citric acid. The nuclei were precipitated by a 4,000 *g* centrifugation at 4°C for 30 min and frozen at −70°C. DNA was isolated, extracted, and counted for [^{3}H]thymidine incorporation as described previously (8). Gel retardation analysis of protein-DNA complexes are performed with an oligonucleotide of the putative DNA binding site, as described previously (7, 8, 24, 37). The sense oligonucleotides were NF-κB (5′-GGGGACTTTCCC-3′) and α-SMA E box (5′-GATCATAAGCAGCTGAACTGCC-3′). Antibodies directed against c-*myb* and NF-κB 65 were obtained from Santa Cruz Biotechnology.

Immunohistochemistry. Liver tissue was immunostained with antisera raised against malondialdehyde-protein adducts as described previously (7, 11, 19). This antiserum is specific from malondialdehyde-lysine adducts (3, 20). A phase-contrast microscope was utilized for hematoxylin/eosin staining and immunohistochemistry with alkaline phosphatase secondary antibodies (Vector Laboratories). Cytochromes utilized were alkaline phosphatase with fast green as counterstain (Sigma Chemical). Negative control samples were processed in parallel under the same conditions, but with omission of the primary antibody.

Synthesis of pentoxifylline metabolite 5. Metabolite 5 of pentoxifylline (1-[3-carboxypropyl]-3,7-dimethylxanthine) and its ethyl ester were synthesized as described elsewhere (12). Briefly, theobromine (2 mmol) was combined with anhydrous K_2CO_3 (2.5 mmol) and dry dimethyl formamide (15 ml), and the mixture was brought to 75°C. The alkyl halide (2.5 mmol) was added and the mixture was stirred at 75°C for 18 h. The reaction mixture was cooled, poured into water (125 ml), and extracted with ethyl acetate (2 × 75 ml). The organic layer was dried over magnesium sulfate and evaporated to yield a white solid, which was triturated with ethyl ether. The resulting solid, analytically pure, was purified further by crystallization. H nuclear magnetic resonance spectrum, elemental analyses, and exact mass data were consistent with the assigned structure.

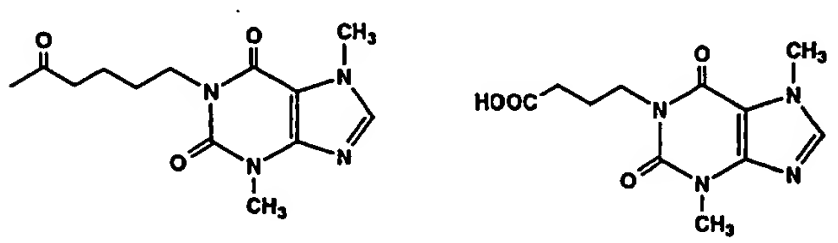
Superoxide assay. Human neutrophils were isolated from heparinized normal donor blood by Histopaque density gradient followed by hypotonic lysis to remove red blood cells. Cells (5×10^6) were suspended in 200 μl Hanks' balanced salt solution without phenol red and containing TNF-α (1 U) and pentoxifylline (100 μM) or metabolite 5 (100 μM). After 20 min, 1 ml of 120 μM cytochrome *c* with 100 nM *N*-formyl-Met-Leu-Phe (FMLP) was added. After a 10-min incubation at 37°C, the optical density (OD_{550}) of the supernatant was determined.

Statistical analysis. Results were expressed as the mean of at least three independent experiments unless stated otherwise. The Student's *t*-test was used to evaluate the differences of the means between groups, with a P value of <0.05 treated as significant.

RESULTS

The role of pentoxifylline [a trisubstituted xanthine derivative (see Fig. 1) that decreases blood viscosity] on stellate cell activation in vivo was examined. Rats were treated with carbon tetrachloride, an hepatotoxin that induces liver lipid peroxidation (3) and stellate cell activation (31).

Stellate cell proliferation was assessed by the incorporation of [^{3}H]thymidine, and activation by the expression of α-SMA (8, 31). The specific activity of the stellate cell DNA, an index of S phase labeling, was determined in all groups after 4 h of labeling with [^{3}H]thymidine at 1:00 PM, to avoid potential variability related to circadian rhythm (8). The dose of [^{3}H]thymidine was given intraperitoneally, as described previously (8). The [^{3}H]thymidine specific activity of the stellate cell DNA increased ~10-fold 48 h after administration of CCl_4 (Fig. 2*A*). Pentoxifylline treatment abolished the proliferation of stellate cells in animals treated with CCl_4 (Fig. 2*A*), suggesting an inhibitory effect of pentoxifylline on stellate cell activation. As depicted in Fig. 2*B*, we found that hepatic stellate cells of control animals (*lane 1*) were activated by treatment with CCl_4 (19), as determined by increased expression of α-SMA on Western blots of freshly isolated stellate cells (*lane 2*). Treatment with pentoxifylline prevented activation of stellate cells induced by CCl_4 (*lane 3*). However, these effects of pentoxifylline could be the result of interfering with the hepatocellular injury induced by CCl_4. Therefore,



Pentoxifylline **Metabolite 5**

Fig. 1. Structure of pentoxifylline and metabolite 5. Pentoxifylline and metabolite 5 are trisubstituted xanthine derivatives. Pentoxifylline is designated chemically as 1-(5-oxohexyl)-3,7-dimethylxanthine, and metabolite 5 is an *N*-1 carboxypropyl derivative of pentoxifylline.

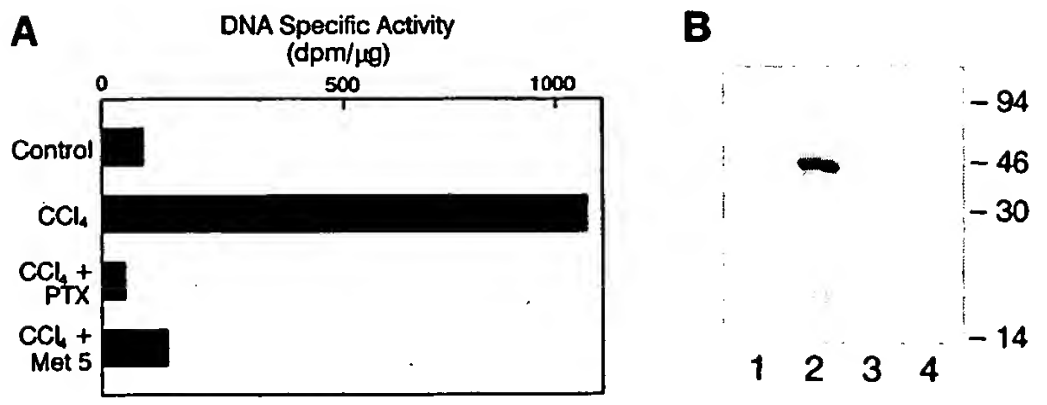


Fig. 2. Pentoxifylline (PTX) and metabolite 5 (Met 5) inhibit stellate cell proliferation and activation in vivo. *A*: DNA specific activity, an index of S phase labeling, was determined in DNA (10 μg) purified from stellate cells isolated from at least 4 rats of each experimental group, as described in METHODS. $P < 0.05$ for CCl_4 compared with all other groups. SE was <20% of the mean for all conditions. dpm, Disintegrations/min. *B*: α-smooth muscle actin (α-SMA) expression was analyzed in stellate cell lysates (10 μg of protein) isolated from control (*lane 1*), CCl_4 (*lane 2*), CCl_4 + pentoxifylline (*lane 3*), and CCl_4 + metabolite 5 (*lane 4*) animals by Western blot as described in METHODS. α-SMA was detected only in CCl_4 animals. Molecular markers are shown.

the degrees of hepatocellular injury and lipid peroxidation were determined in these animals.

Although the histological degree of hepatocellular necrosis (at 48 h) was similar in the CCl_4 and CCl_4 + pentoxifylline groups, as indicated by liver staining with hematoxylin/eosin (not shown), pentoxifylline reduced significantly ($P < 0.05$) the release of alanine aminotransferase into the blood at 24, 36, and 48 h (Table 1), indicating a protective effect of pentoxifylline in this animal model of toxic hepatitis. The degree of hepatic lipid peroxidation was comparable in both groups of animals (Fig. 3). Protein adducts with malondialdehyde, a product of lipid peroxidation, were detected using specific antibodies against malondialdehyde-lysine epitopes, as reported previously (3, 19, 20). In contrast to the livers of control animals (Fig. 3*A*), enhanced lipid peroxidation was comparable at 48 h in zones 2 and 3 of the hepatic acini in animals treated with CCl_4 alone (Fig. 3*B*) or with the addition of pentoxifylline (Fig. 3*C*).

Because oxidative stress increases NF-κB activity (18) and NF-κB plays important roles in the regulation of stellate cell activation by collagen type I and TGF-α (24), we analyzed the potential role of NF-κB regulation in stellate cell activation in animals treated with CCl_4. Stellate cell activation in vivo was also associated with the nuclear translocation and activation of NF-κB, as detected by gel shift analysis. As shown in Fig. 4*A*, the binding of stellate cell nuclear extracts to a NF-κB cognate oligonucleotide was low in quiescent cells from control animals (*lane 2*) but increased significantly following stellate cell activation after treatment with CCl_4 (*lane 3*). The complex of ^{32}P-labeled NF-κB oligonucleotides and nuclear extracts from activated stellate cells was competed with by a NF-κB cognate oligonucleotide (*lane 6*) but not by unrelated oligonucleotides (not shown). Of interest, pentoxifylline treatment prevented not only stellate cell proliferation and activation (Fig. 2) but also NF-κB nuclear activity (Fig. 4*A*, *lane 4*) induced by CCl_4.

Table 1. *Effects of pentoxifylline on serum alanine aminotransferase levels in CCl_4-induced hepatotoxicity*

Group	Serum Alanine Aminotransferase, IU/l		
	24 h	36 h	48 h
Control	74 ± 4	73 ± 3	65 ± 7
CCl_4	752 ± 144	577 ± 38	397 ± 5
CCl_4 + P	259 ± 37	294 ± 66	231 ± 56

Values are means ± SE. Serum alanine aminotransferase values were determined in control, CCl_4, and CCl_4 + pentoxifylline (P) at 24, 36, and 48 h after injection of mineral oil or CCl_4. Pentoxifylline was given as described in METHODS. $P < 0.05$ for CCl_4 + P vs. CCl_4 at all time points.

Because c-*myb* is an important inducer of proliferation in cultured hematopoietic, smooth muscle, and stellate cells (1, 24, 33), we tested whether c-*myb* expression plays a role in the activation of stellate cells in vivo. As expected, the critical promoter E box of the α-SMA gene (6) formed complexes with nuclear extracts from freshly isolated activated stellate cells from CCl_4-treated animals (Fig. 4*B*, *lane 3*), but not with nuclear extracts of freshly isolated quiescent stellate cells from control animals (*lane 2*). Treatment of CCl_4 animals with pentoxifylline prevented the formation of a complex between the α-SMA-E box and stellate cell nuclear extracts (*lane 4*), an essential step in the activation of the α-SMA gene. Relevant to this study, the protein-DNA complexes were disrupted by polyclonal c-*myb* antibodies without the formation of a supershift (Fig. 4*B*, *lane 6*), as described for stellate cells in culture (24), but not by a NF-κB cognate oligonucleotide (*lane 7*). Preimmune serum did not affect the protein-DNA complexes (not shown). These results strongly suggest a role for c-*myb* (and presumably an indirect effect of NF-κB) on α-SMA gene expression, a hallmark of stellate cell activation (31).

To understand the mechanisms responsible for the blocking effects of pentoxifylline on stellate cell activation, we analyzed whether they are related to its role as a cyclic nucleotide phosphodiesterase inhibitor. A major metabolite of pentoxifylline, metabolite 5 (38), was synthesized as an ethyl ester prodrug (12) (Fig. 1) and found to lack the inhibitory activity of phosphodiesterase from human neutrophils (12).

To test the biological activities of pentoxifylline and metabolite 5 independently of hepatocellular necrosis, we studied their effects on the proliferation of primary rat stellate cells. Both pentoxifylline and metabolite 5

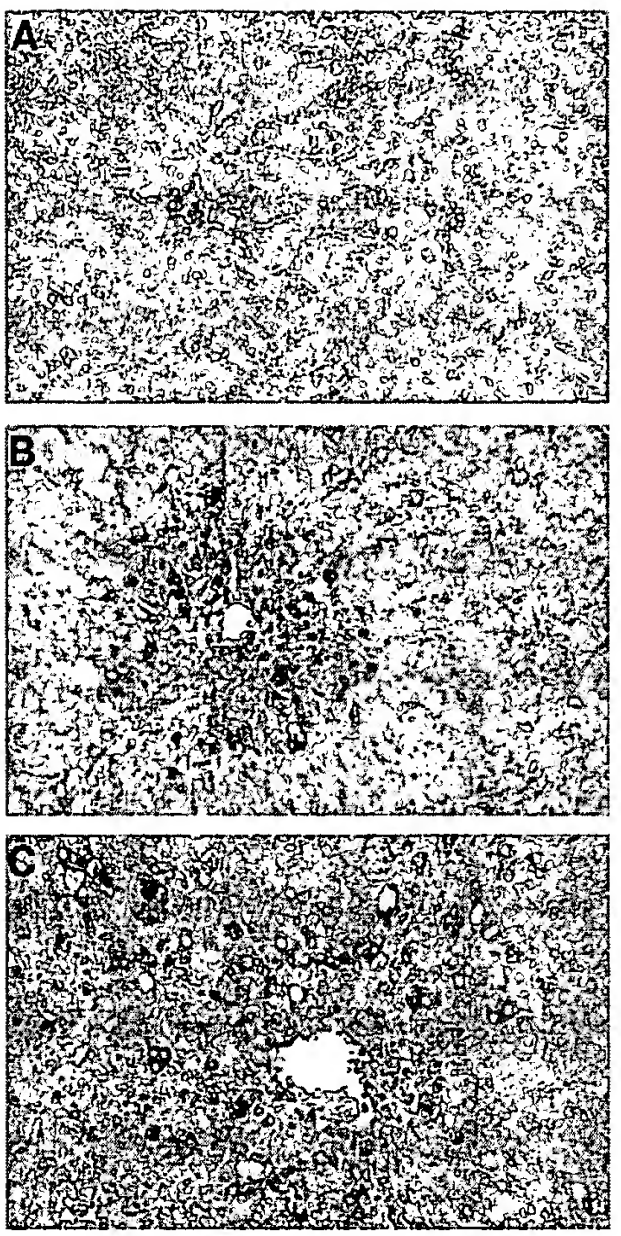


Fig. 3. Induction of hepatic lipid peroxidation by CCl_4. Animals were studied 48 h after mineral oil (*A*) or CCl_4 (*B* and *C*) treatment, and liver tissue was processed for malondialdehyde (MDA)-protein adduct immunohistochemistry using specific antibodies against MDA-lysine epitopes as described in METHODS. *A*: no adducts were detected in mineral oil-treated animals. MDA-protein adducts are detected to a comparable degree in the cytosol of hepatocytes in zones 2 and 3 of the acinus in the livers from CCl_4 (*B*) and CCl_4 + pentoxifylline (*C*). Magnification ×100.

blocked stellate cell proliferation induced by collagen type I matrix (Fig. 5*A*), which is mediated by an oxidative stress cascade (24). These results indicate that pentoxifylline and metabolite 5 are able to block the oxidative stress cascade induced by collagen type I matrix (24) acting directly on stellate cells. Pentoxifylline and metabolite 5 also inhibited the proliferation of NIH/3T3 fibroblasts induced by serum with a half-maximal inhibitory concentration (~100 μM) (Fig. 5*B*) similar to that observed in stellate cells.

Next, we determined whether pentoxifylline inhibits cAMP phosphodiesterase activity in hepatic stellate cells in vivo, leading to an increase in protein kinase A-mediated phosphorylation. This signal-transduction pathway triggers site-specific phosphorylation of the nuclear transcription factor CREB on Ser^{133} (17). Therefore, the induction of CREB phosphorylation at Ser^{133} was analyzed in nuclear extracts from freshly isolated stellate cells using an antibody against the activated, phosphorylated form of CREB (21). As depicted in Fig. 6, treatment with pentoxifylline markedly increased the expression of CREB-$PSer^{133}$ (43 kDa) (*lane 3*), which was also detected in stellate cells from control groups in longer exposures (not shown) (21), but not in stellate cells from CCl_4 (*lane 2*) or CCl_4 + metabolite 5 (*lane 4*) groups. In addition to recognizing CREB, anti-CREB-$PSer^{133}$ detected two other proteins. These are most likely members of the CREB-activating transcription factor (ATF) family, ATF-1 and cAMP response element modulator protein (CREM), that have phosphoacceptor sequences similar to that of CREB-$PSer^{133}$ (21). Nuclear extracts of stellate cells isolated from control (Fig. 6, *lane 1*) and CCl_4 + metabolite 5 (*lane 4*) animals also contain small amounts of phosphorylated ATF-1 (38 kDa) and CREM (30 kDa). Neither CREB-$PSer^{133}$ nor phosphorylated members of the CREB-ATF family were detected in stellate cell nuclear extracts from CCl_4-treated animals (*lane 2*). This study indicates that pentoxifylline, but not metabolite 5, displays cAMP phosphodiesterase inhibitory effects on hepatic stellate cells in vivo.

In subsequent experiments, we analyzed the effects of metabolite 5 on CCl_4-induced hepatotoxicity and stellate cell activation. Treatment with metabolite 5 prevented to a considerable extent the proliferation (Fig. 2*A*) and activation (Fig. 2*B*, *lane 4*) of stellate cells induced by the administration of CCl_4. Moreover, treatment of CCl_4 animals with metabolite 5 also prevented the molecular changes characteristic of stellate cell activation, including enhanced nuclear activities of NF-κB and c-*myb* (Fig. 4). These findings suggest that the cyclic nucleotide phosphodiesterase inhibitory activity of pentoxifylline is not indispensable to block stellate cell activation or proliferation.

Coculture experiments of hepatocytes and stellate cells treated with CCl_4 indicate that hepatocytes exert a paracrine stimulation of both lipid peroxidation and collagen gene expression on stellate cells (3). Because pentoxifylline and metabolite 5 interfere with the oxidative stress cascade in CCl_4-induced hepatic injury (Table 1) as well as in primary rat stellate cells (Fig. 5*A*), we analyzed whether a similar effect on oxidative stress occurs in activated neutrophils. Both pentoxifylline and metabolite 5 prevented the production of superoxide in human neutrophils treated with TNF-α and FMLP (Fig. 7), known inducers of oxidative stress in these cells (2, 18, 23).

The redox state of the cell may alter the DNA binding affinity of activator protein-1 (AP-1) factors and c-*myb* (39, 40). The modulation of c-*myb* is probably mediated through a conserved cysteine amino acid motif (KQC43R) within the DNA binding domain (40). Therefore, we determined whether 1,4-dithiothreitol (DTT) would normalize the increased binding of stellate cell nuclear extracts from CCl_4-treated animals to the α-SMA-E box. The addition of DTT to the nuclear extracts as described previously (40) (Fig. 4*C*, *lane 4*) normalized the increased DNA-protein complex (compare *lanes 2* and *3*), suggesting that the critical mechanism involves oxidation of a transcription factor, presumably the DNA binding domain of c-*myb*.

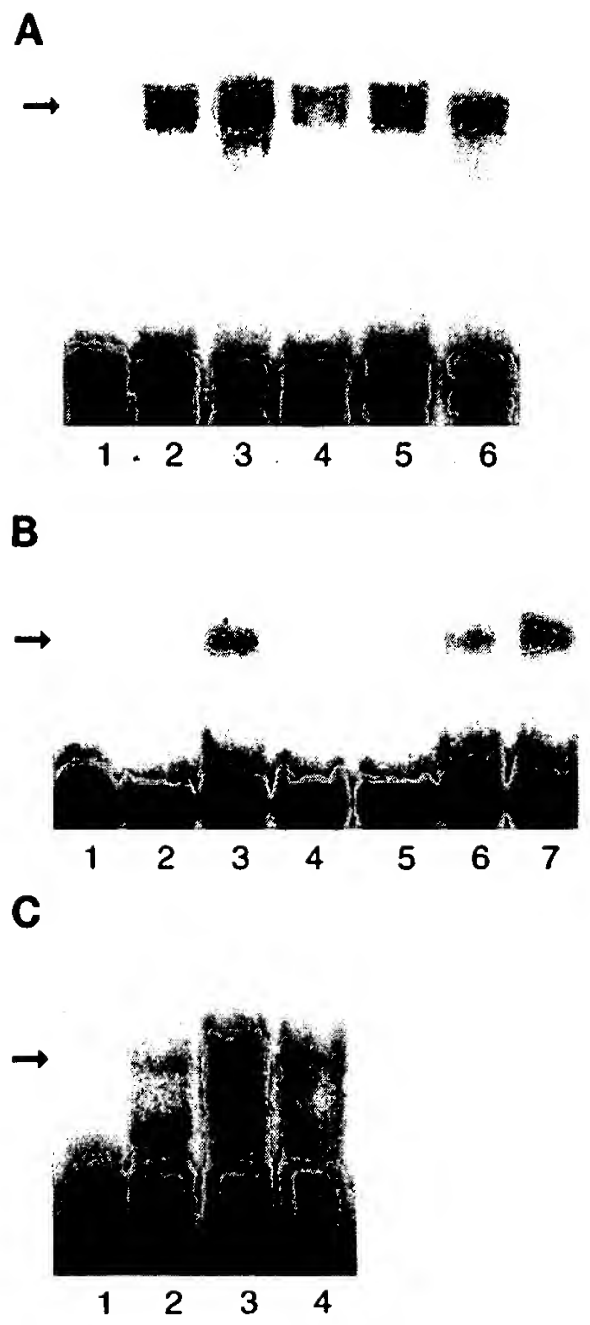


Fig. 4. Pentoxifylline and metabolite 5 block the increase in nuclear factor (NF)-κB and α-SMA binding activities of activated stellate cells. Mobility shift analysis of stellate cell nuclear extracts. Equal amounts of nuclear extracts (3 μg of DNA) were incubated with 1 ng of ^{32}P-labeled-NF-κB (*A*) or ^{32}P-labeled-α-SMA-E box (*B* and *C*) oligonucleotides. The DNA-protein complexes were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel. Position of the bound DNA is indicated by arrows. Some samples were incubated with specific antibodies, unlabeled oligonucleotide, or 1,4-dithiothreitol (DTT), as indicated by +. *A*: representative samples of control (*lane 2*), CCl_4 (*lane 3*), CCl_4 + pentoxifylline (*lane 4*), CCl_4 + metabolite 5 (*lane 5*), and CCl_4 + NF-κB oligonucleotide (*lane 6*). On *lane 1*, ^{32}P-labeled-NF-κB probe was processed without nuclear extracts. *B*: representative samples of control (*lane 2*), CCl_4 (*lane 3*), CCl_4 + pentoxifylline (*lane 4*), CCl_4 + metabolite 5 (*lane 5*), CCl_4 + c-*myb* antibodies (*lane 6*), and CCl_4 + NF-κB oligonucleotide (*lane 7*). On *lane 1*, ^{32}P-labeled-α-SMA-E box probe was processed without nuclear extracts. *C*: representative examples of control (*lane 2*), CCl_4 (*lane 3*), and CCl_4 + DTT (*lane 4*). On *lane 1*, ^{32}P-labeled-α-SMA-E box probe was processed without nuclear extracts.

DISCUSSION

In this study, we have characterized some of the molecular mechanisms involved in the activation of stellate cells in vivo, an important step in hepatic fibrogenesis (14, 15). Moreover, we have identified a cellular pathway leading to stellate cell activation that is blocked by pentoxifylline. The inhibitory activity of pentoxifylline on cyclic nucleotide phosphodiesterases is not required to block stellate cell activation.

Our results suggest a critical role of NF-κB and c-*myb* on stellate cell activation in vivo, given that during stellate cell activation the nuclear activities of NF-κB and c-*myb* are increased and that these molecular changes and stellate cell activation are both blocked by pentoxifylline and its metabolite 5. Also, we determined that in activated stellate cells from CCl_4-treated animals, c-*myb* contributes substantially to nuclear binding to the key E box within the promoter of the α-SMA gene. These findings strongly suggest that c-*myb* is the molecular mediator of oxidative stress on stellate cell activation and that it binds to the critical E box of the α-SMA gene (6), the expression of which is intrinsic to the activated phenotype of stellate cells (31).

Pentoxifylline and the metabolite 5 inhibited the enhanced NF-κB and c-*myb* activities of stellate cells in CCl_4-induced hepatic injury. The dose of pentoxifylline used was not toxic to the animals, as judged by their normal behavior, lack of hepatotoxicity, and preservation of cAMP-mediated phosphorylation of stellate cells. However, pentoxifylline ameliorated the release of alanine aminotransferase, indicating a protective effect in CCl_4-induced hepatotoxicity. Given that pentoxifylline inhibits the lipopolysaccharide-induced production of TNF-α (25, 32), it might inhibit this critical pathway in Kupffer cells, resulting in decreased leakage of alanine aminotransferase (10). However, it is improbable that the inhibitory effects of pentoxifylline and metabolite 5

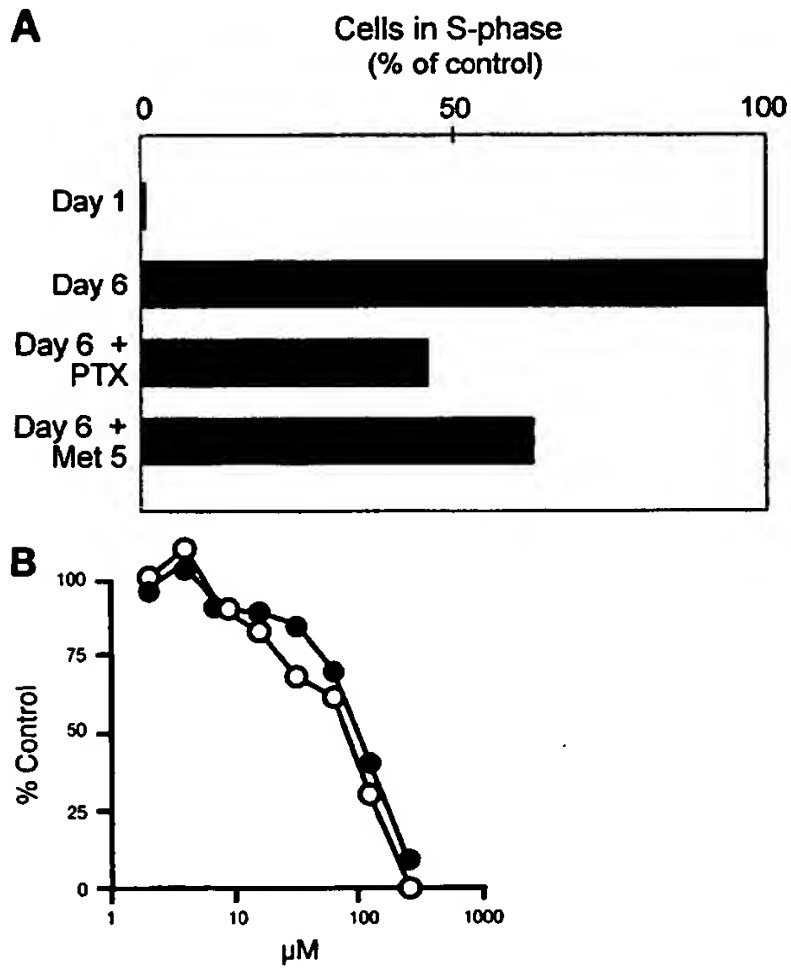


Fig. 5. Pentoxifylline and metabolite 5 inhibit proliferation of primary rat stellate cells. *A*: [^{3}H]thymidine incorporation into DNA (2 μCi of 6-[^{3}H]thymidine/P-60 plate for 3 h), an index of S phase, was determined in primary rat stellate cells cultured on a collagen type I matrix as described in METHODS. $P < 0.05$ for pentoxifylline and metabolite 5. SE was <20% of mean for all conditions. *B*: [^{3}H]thymidine incorporation into DNA was determined in NIH/3T3 fibroblasts as described in METHODS. $P < 0.05$ for pentoxifylline (○) and metabolite 5 (●). SE was <30% of mean for all conditions.

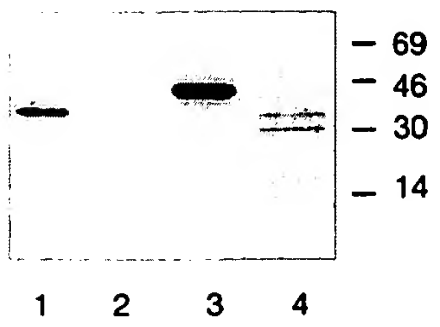


Fig. 6. Pentoxifylline, but not metabolite 5, stimulates protein kinase A-mediated phosphorylation. Phosphorylation of cAMP-responsive element binding protein (CREB) at Ser^{133} was analyzed in stellate cell nuclear extracts (5 μg of protein) from control (*lane 1*), CCl_4 (*lane 2*), CCl_4 + pentoxifylline (*lane 3*), and CCl_4 + metabolite 5 (*lane 4*) animals by Western blot using antibodies against CREB-$PSer^{133}$ as described in METHODS. CREB-$PSer^{133}$ was detected only in CCl_4 (*lane 2*) animals. Molecular markers are shown in kDa.

on stellate cell activation/proliferation in vivo are mediated by inhibition of TNF-α production. TNF-α has no (or a small) stimulatory effect on stellate cell proliferation and no effect on stellate cell activation (9). Finally, collagen gene expression in stellate cells is inhibited by TNF-α, an effect incongruent with a potential role of TNF-α in stellate cell activation (9).

Hepatocytes can exert a paracrine stimulation of both lipid peroxidation and collagen gene expression on stellate cells (3). Potential mediators of this model of oxidative stress in stellate cells include free radicals, reactive aldehydes, or cytokines produced by hepatocytes in response to CCl_4. In this context, we have demonstrated the propagation of the oxidative stress cascade to stellate cells after CCl_4 treatment, as judged by activation of NF-κB and c-*myb*. The increase in this oxidative stress cascade within stellate cells suggests that this mechanism may be responsible for the molecular changes leading to stellate cell activation, as predicted from studies in cultured stellate cells (24). Several cytokines stimulate stellate cell activation, including platelet-derived growth factor (PDGF) and TGF-α (30). Interestingly, the induction of stellate cell activation by TGF-α utilizes an oxidative pathway, because it can be blocked with antioxidants (24). Similarly, PDGF requires H_2O_2 generation for signal transduction in vascular smooth muscle cells (35). In addition, Ras-transformed NIH/3T3 fibroblasts produced large amounts of superoxide, and their mitogenic activity was inhibited with the antioxidant *N*-acetyl-L-cysteine (22). Pentoxifylline also inhibited proliferation of NIH/3T3 fibroblasts. These studies support the notion that cytokine and oxidative stress pathways converge in the activation of stellate cells (24) and that pentoxifylline blocks these pathways, as suggested for superoxide production by neutrophils (13).

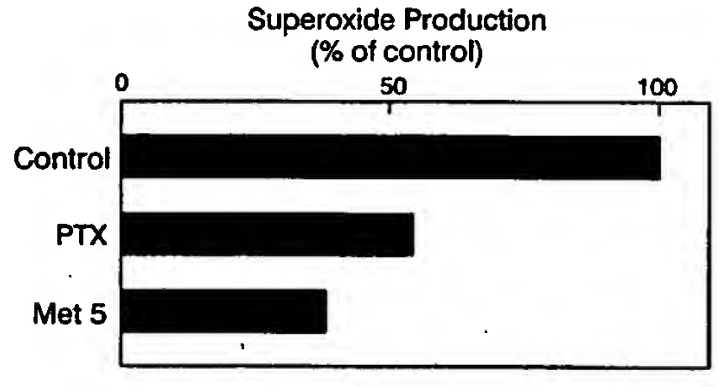


Fig. 7. Superoxide production in human neutrophils is prevented by pentoxifylline and metabolite 5. Induction of superoxide production by TNF-α and *N*-formyl-Met-Leu-Phe in human neutrophils (control), as described in METHODS, was prevented by pentoxifylline (100 μM) and metabolite 5 (100 μM). Values are percentage of control. $P < 0.05$ for pentoxifylline and metabolite 5. SE was <20% of mean for all conditions.

The binding of c-*myb* (and other transcription factors) to cognate DNA sequences can be modulated by the redox state of the cell (39, 40). We found that the addition of DTT, a reducing agent, normalized the increased α-SMA-E box binding activities of stellate cell nuclear extracts from CCl_4-treated animals, suggesting an oxidative modification of a critical binding factor, such as c-*myb*. Future studies should assess whether this oxidative cascade involves the nuclear redox factor Ref-1 (39), which functions as a DNA repair enzyme and modulates the DNA binding activity of several transcription factors (40). Although xanthine and hypoxanthine can initiate the production of hydroxyperoxide radicals in the presence of xanthine oxidase (2, 23), they are also capable by themselves of scavenging these free radicals (2). Given that pentoxifylline and metabolite 5 are substituted xanthines (12, 38), it is likely that this structure is responsible for blocking the oxidative stress cascade in the liver of animals treated with CCl_4 as well as in primary stellate cells. Indeed, we demonstrated that pentoxifylline and metabolite 5 prevent the formation of superoxide induced by TNF-α and FMLP, indicating that both act as scavengers of free radicals.

Increased cAMP-dependent protein kinase activity in stellate cells treated with pentoxifylline was evident by the phosphorylation of CREB on Ser^{133}, a classic phosphoacceptor for protein kinase A (17, 21). This assay also documented the inability of metabolite 5 to affect phosphodiesterase activity in vivo. Altogether, these results indicate that the effects of pentoxifylline on stellate cell activation are independent of its phosphodiesterase inhibitory activity and are most likely related to its ability to block the propagation of oxidative stress and the induction of NF-κB and c-*myb* within the stellate cells. Studies in animals with targeted deletions of NF-κB or c-*myb* may establish the role of these genes in stellate cell activation in vivo.

Our study provides insights into the molecular mechanisms leading to hepatic stellate cell activation in vivo and the blocking effects of pentoxifylline on these pathways. The findings presented here should facilitate potential therapeutic approaches for hepatic fibrosis, a major contributor to the morbidity and mortality of patients with chronic liver diseases.

We are grateful to M. Buck for valuable suggestions. We thank K. Pak for technical assistance and L. Masse for the preparation of this manuscript.

This study was supported in part by National Institutes of Health Grants GM-23200, DK-38652, DK-46971, and GM-47165 and by grants from the Department of Veterans Affairs and the American Liver Foundation. K. S. Lee was supported by a grant from Yonsei University College of Medicine (Seoul, South Korea), and K. Houglum was supported by a Clinical Investigator Award (DK-02265).

Address for reprint requests: M. Chojkier, Dept. of Medicine and Center for Molecular Genetics (9111-D), Univ. of California, San Diego, CA 92161.

Received 11 July 1997; accepted in final form 4 August 1997.

REFERENCES

1. **Badiani, P., P. Corbella, D. Kioussis, J. Marvel, and K. Weston.** Dominant interfering alleles define a role for c-*myb* in T-cell development. *Genes Dev.* 8: 770–782, 1994.
2. **Becker, B. F., N. Reinholz, B. Leipert, P. Raschke, B. Permanetter, and E. Gerlach.** Role of uric acid as an endogenous radical scavenger and antioxidant. *Chest* 100: 176S–181S, 1991.
3. **Bedossa, P., K. Houglum, C. Trautwein, A. Holstege, and M. Chojkier.** Stimulation of collagen α_1(I) gene expression is associated with lipid peroxidation in hepatocellular injury. A link to tissue fibrosis? *Hepatology* 19: 1262–1271, 1994.
4. **Berman, B., J. Weitzerbin, J. Sanceau, G. Merlin, and M. R. Duncan.** Pentoxifylline inhibits certain constitutive and tumor necrosis factor-α-induced activities of human normal dermal fibroblasts. *J. Invest. Dermatol.* 98: 706–712, 1992.
5. **Biswas, D. K., C. M. Ahlers, B. J. Dezube, and A. B. Pardee.** Cooperative inhibition of NF-kB and Tat-induced superactivation of human immunodeficiency virus type 1 long terminal repeat. *Proc. Natl. Acad. Sci. USA* 90: 11044–11048, 1993.
6. **Blank, R. S., T. C. McQuinn, K. C. Yin, M. M. Thompson, K. Takeyasu, R. J. Schwartz, and G. K. Owens.** Elements of the smooth muscle α-actin promoter required in *cis* for transcriptional activation in smooth muscle. *J. Biol. Chem.* 267: 984–988, 1992.
7. **Buck, M., and M. Chojkier.** Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *EMBO J.* 15: 1753–1765, 1996.
8. **Buck, M., H. Turler, and M. Chojkier.** LAP, a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. *EMBO J.* 13: 851–860, 1994.
9. **Chojkier, M.** Regulation of collagen gene expression. In: *Liver Growth and Regeneration*, edited by A. Strain and A. Diehl. London: Chapman & Hall, 1997, p. 430–450.
10. **Chojkier, M., and J. Fierer.** D-Galactosamine hepatotoxicity is associated with endotoxin-sensitivity and mediated by lymphoreticular cells in mice. *Gastroenterology* 88: 115–121, 1985.
11. **Chojkier, M., K. Houglum, J. Solis-Herruzo, and D. A. Brenner.** Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation? *J. Biol. Chem.* 264: 16957–16962, 1989.
12. **Cottam, H. B., H. Shih, L. R. Tehrani, D. B. Wasson, and D. A. Carson.** Substituted xanthines, pteridinediones, and related compounds as potential antiinflammatory agents. Synthesis and biological evaluation of inhibitors of tumor necrosis factor-α. *J. Med. Chem.* 39: 2–9, 1995.
13. **Crouch, S. P. M., and J. Fletcher.** Effect of ingested pentoxyfilline on neutrophil superoxide anion production. *Infect. Immun.* 60: 4504–4509, 1992.
14. **Davis, B. H., B. M. Pratt, and J. A. Madri.** Retinol and extracellular collagen matrices modulate hepatic Ito cell collagen phenotype and cellular retinol binding protein levels. *J. Biol. Chem.* 262: 10280–10286, 1987.
15. **Friedman, S. L., F. J. Roll, J. Boyles, D. M. Arenson, and D. M. Bissell.** Maintenance of differentiated phenotype of cultured rat hepatic stellate cells by basement membrane matrix. *J. Biol. Chem.* 264: 10756–10762, 1989.
16. **Friedman, S., F. Roll, J. Boyles, and D. Bissell.** Hepatic stellate cells: the principal collagen-producing cells of the liver. *Proc. Natl. Acad. Sci. USA* 82: 8681–8685, 1985.
17. **Gonzalez, G., and M. R. Montminy.** Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59: 675–680, 1989.
18. **Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P. Baeuerle.** Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature* 365: 182–185, 1993.
19. **Houglum, K., M. Buck, J. Alcorn, S. Contreras, P. Bornstein, and M. Chojkier.** Two different *cis*-acting regulatory regions direct cell-specific transcription of the collagen α_1(I) gene in hepatic stellate cells and in skin and tendon fibroblasts. *J. Clin. Invest.* 96: 2269–2276, 1995.
20. **Houglum, K., M. Filip, J. L. Witztum, and M. Chojkier.** Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. *J. Clin. Invest.* 86: 1991–1998, 1990.
21. **Houglum, K., K. S. Lee, and M. Chojkier.** Proliferation of hepatic stellate cells is inhibited by phosphorylation of CREB on serine 133. *J. Clin. Invest.* 99: 1322–1328, 1997.
22. **Irani, K., Y. Xia, J. L. Zweier, S. J. Sollott, C. J. Der, E. R. Fearon, M. Sundaresan, T. Finkel, and P. J. Goldschmidt-Clermont.** Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* 275: 1649–1652, 1997.
23. **Koenderman, L., M. Yazdanbakhsh, D. Roos, and A. J. Verhoeven.** Dual mechanisms in priming of the chemoattractant-induced respiratory burst in human granulocytes. *J. Immunol.* 142: 623–628, 1989.
24. **Lee, K. S., M. Buck, K. Houglum, and M. Chojkier.** Activation of hepatic stellate cells by TGFα and collagen type I is mediated by oxidative stress through c-*myb* expression. *J. Clin. Invest.* 96: 2461–2468, 1995.
25. **Levi, M., H. ten Cate, K. A. Bauer, T. van der Pool, T. S. Edgington, H. R. Büller, S. J. H. van Deventer, C. E. Hack, J. Wouter ten Cate, and R. D. Rosenberg.** Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J. Clin. Invest.* 93: 114–120, 1994.
26. **Maher, J. J., and R. F. McGuire.** Extracellular matrix gene expression increases preferentially in rat stellate cells and sinusoidal endothelial cells during hepatic fibrosis in vivo. *J. Clin. Invest.* 86: 1641–1648, 1990.
27. **McCord, J. M.** Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* 312: 159–163, 1985.
28. **Nicholson, C. D., R. A. J. Challis, and M. Shahid.** Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.* 12: 19–27, 1991.
29. **Peterson, T. C.** Pentoxifylline prevents fibrosis in an animal model and inhibits platelet-derived growth factor-driven proliferation of fibroblasts. *Hepatology* 17: 486–493, 1992.
30. **Pinzani, M., L. Gesulado, G. Sabbah, and H. Abboud.** Effects of platelet derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat storing cells. *J. Clin. Invest.* 84: 1786–1793, 1989.
31. **Rockey, D., J. Boyles, G. Gabbiani, and S. Friedman.** Rat hepatic stellate cells express smooth muscle actin upon activation in vivo and in culture. *J. Submicrosc. Cytol. Pathol.* 24: 193–203, 1992.
32. **Schandené, L., P. Vandenbussche, A. Crusiaux, M.-L. Alègre, D. Abramowicz, E. Dupont, J. Content, and M. Goldman.** Differential effects of pentoxifylline on the production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by monocytes and T cells. *Immunology* 76: 30–34, 1992.
33. **Simons, M., and R. D. Rosenberg.** Antisense nonmuscle myosin heavy chain and c-*myb* oligonucleotides suppress smooth muscle cell proliferation in vitro. *Circ. Res.* 70: 835–843, 1992.
34. **Solis-Herruzo, J. A., D. A. Brenner, and M. Chojkier.** Tumor necrosis factor-α inhibits collagen gene transcription and collagen synthesis in cultured human fibroblasts. *J. Biol. Chem.* 263: 5841–5845, 1988.
35. **Sundaresan, M., Z.-X. Yu, V. J. Ferrans, K. Irani, and T. Finkel.** Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270: 296–299, 1995.
36. **Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier.** Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* 364: 544–547, 1993.
37. **Trautwein, C., P. van der Geer, M. Karin, T. Hunter, and M. Chojkier.** Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA-recognition elements. *J. Clin. Invest.* 93: 2554–2561, 1994.
38. **Ward, A., and S. P. Clissold.** Pentoxifylline. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic efficacy. *Drugs* 34: 50–97, 1987.
39. **Xanthoudakis, S., and T. Curran.** Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J.* 11: 653–665, 1992.
40. **Xanthoudakis, S., G. Miao, F. Wang, Y. C. E. Pan, and T. Curran.** Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J.* 11: 3323–3335, 1992.